EFFECT OF DIAGNOSTIC ULTRASOUND ON THE DEVELOPING CHICK EMBRYO

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EFFECT OF DIAGNOSTIC ULTRASOUND

ON THE DEVELOPING CHICK EMBRYO

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SYMBOLS

°C	Degrees centigrade
cm^2	Square centimeters
$\overline{F}$	Force
gm.	Grams
Hz.	Hertz (cycles per second)
M.	Molar
MHz.	Megahertz
m/s	Meters per second
mg.	Milligrams
ml.	Milliliters
mW.	Milliwatts
N.	Normal
$\overline{V}$	Velocity
W	Watts
ΔT	Temperature increase in °C

SUMMARY

Ultrasound has become an increasingly useful tool to the physician. One of the reasons for this is the supposed safety with which non-invasive data can be obtained. Although current publications indicate that no harmful effects exist, only gross measurements have been made. No effects on embryonic development have been studied.

This study investigated the possible harmful effects of pulsed diagnostic ultrasound (under 50 mW/cm^2) on developing chick embryos. A 2.0 MHz transducer and a commercially available clinical ultrasound diagnostic unit were used. Two studies were performed.

In the first experiment fertile White Leghorn eggs were incubated for two days, insonated at one of two intensities (15 mW/cm^2 or 49 mW/cm^2) for three minutes through the shell and allowed to incubate for two more days before analysis. Using the total number of somite pairs as a growth indicator, the insonated embryos showed a small but consistent growth retardation when compared with controls which had been subjected to a dummy transducer. This experiment was repeated a total of six times with similar results.

In the second study fertile eggs were treated as before except that they were allowed to develop in the incubator until they were four, eight, or twelve days old. These embryos were then dissected and the brain and trunk regions were assayed separately for total RNA, DNA, and protein content. The results indicated that by eight days the brains showed a measurable decrease in total protein content.

Although the statistical significances were not extremely large due to the high degree of variability of the embryos, the consistency of these results clearly indicate some effect due to ultrasound in the diagnostic range. Only further study can determine if these effects are biologically significant.

CHAPTER I

INTRODUCTION

Background

Ultrasound has become an increasingly useful tool to the physician. In recent years, it has entered into virtually all areas of treatment and diagnosis [1-7]. One of the reasons for this is the supposed safety with which non-invasive data can be obtained, as opposed to ionizing radiation, such as x-rays, the harmful effects of which are now known.

Ultrasound is usually considered to be the range above human hearing, about 20,000 Hz. In diagnostic work today, the range is commonly between 1 and 10 MHz. It has been shown that high intensities (1-15 W/cm^2) can produce biological alterations [8-16].

Of particular interest, however, are the low level intensities (1-50 mW/cm^2) which are being widely used today, especially in pulsed ultrasound, where each pulse may have a peak intensity of 1 W/cm^2 or more. However, since these peaks are only one microsecond in duration, followed by long periods of inaction (one millisecond or more), the time-average intensity is still in the low level range.

The biological effects of ultrasound were first investigated by Wood and Loomis in 1928 [17]. Since that time, there have been countless reports to show that no harm is caused at these low intensity levels [18-30]. However, a careful examination of these reports show

that the question of safety is far from answered in the minds of the investigators. Most of the reports investigated the safety of diagnostic pulsed ultrasound as used in obstetrical examinations, since it is logical that damage would be most pronounced in a developing embryo.

Freimanis [18], after stating that there is no present reason to be concerned about such effects, said, "Nevertheless, it would be quite desirable to obtain some more studies of the possible effects of diagnostic ultrasonic radiation on the earliest stages of embryonic and fetal development."

Donald et al [19] said similarly

> I would suggest, however, examining litters that have been irradiated with ultrasound in utero and put through intelligence testing subsequently, such as being made to learn and unlearn simple tricks. Their performance would be compared with control litters on a statistical basis ... let us not forget it took forty years to find out the harmful results from ionizing radiations. Everyone of us is exposed to a real condemnation by history if we don't get this problem sorted out pretty quickly.

Kohorn et al [20], after showing no change in the EEG's of insonated newborn babies, still said, "The possibility that abnormal electrical activity might be associated with exposure to diagnostic ultrasound has not been investigated."

A statement in a summary in Lancet [21] on the safety of obstetrical ultrasonic investigations said

> So far no adverse effects have been recorded from the use of sonar but no one who uses it should be unaware of the possible types of damage, which are: (1) structural damage to already formed organs and to organs not fully developed; (2) behavioral changes in the organism and disorders of functions; and perhaps most sinister of all, (3) the possibility of genetic damage - teratogenesis and mutagenesis.

Bobrow et al [22], who was disproving the genetic damage alledged by MacIntosh et al [31], still said;

> Nevertheless, further work on this subject, combined with studies of chromosome damage in infants and fetuses exposed in utero to ultrasonic irradiation are clearly needed. It is urgently necessary, not only to detect any possible harmful effects of this widely used diagnostic technique, but also if no such effects exist, to remove any possible stigma from a most useful clinical tool.

Several studies have been published recently which supposedly investigated the low intensity effect on embryonic tissues. The results of these experiments are far from complete. Smyth [23] used adult rats, not fetal rats, when he reported no change due to low intensities on rat brain tissue. Dunn [24] simulated embryonic tissue by using newborn animals to get his threshold data. This study, therefore, really did not tell what effects would be caused in embryos which were developing much faster than neonatal subjects.

Andrew [25], using frog and perch spawn, Woodward et al [26], using mice, and Hellman et al [27], actually using pregnant women, saw no change in gestation time of offspring, but only gross observations were made in each case. Therefore, these studies still left several questions unanswered about diagnostic levels of ultrasound and its effect on the developing embryo, especially in the first trimester when the development is fastest.

Purpose of Research

It was the purpose of this research to irradiate developing chick embryos with diagnostic levels of pulsed ultrasound and measure changes, if any, in these embryos. The parameters studied included

the total number of somite pairs and a biochemical assay of RNA, DNA and total protein of the head and trunk portions of these embryos. All of these parameters are growth indicators. In addition, a test facility was designed and fabricated to determine if the output of the ultrasonic transducer was actually in the diagnostic range and to quantitate the average output.

Biological Model

White Leghorn chick eggs were selected as a model because they offered several advantages to the experiment. There is an abundance of documentation of the developmental stages of the chick embryo since they are a classical life form for embryological study. The embryos are self-contained so no feeding or cage maintenance is required. In addition, fertile eggs are inexpensive, readily available from a local source, easily handled, and require little in the way of support facilities. Therefore, large quantities could be utilized both as tests and controls.

All fertile eggs used for these experiments were purchased from Chiks of Dixie, a Decatur, Georgia firm. This egg source had the added benefit that all eggs used were not only from the same breed of chicken, but from the same strain (S-288) and flock (#2), thus reducing variability of the embryos. All eggs were considered to be zero hours old as received. This assumption was not quite valid since variations exist between eggs due to several factors. Eggs fully developed in the hen but not laid immediately are, in effect, incubated inside the hen's uterus. Also, some eggs are not fully developed when laid, which

somewhat offsets the previous problem. In addition, the eggs are laid at incubation temperature (38°C) and the embryos continue to develop until they cool to the temperature of their surroundings. Therefore, the eggs laid on warmer days are slightly more developed than those laid on cooler days. Since eggs were delivered only twice weekly, some variability between eggs existed.

After collection, the eggs were placed in coolers (approximately 15°C) until delivery to the laboratory, where they were ready to be placed in the incubator (38°C). Since some eggs had thicker shells than others, some eggs cooled and warmed more readily than others, therefore causing additional variability. This last variation was somewhat reduced by allowing the eggs to stand at room temperature (approximately 25°C) for 24 hours before being placed in the incubator. This allowed all eggs to warm more evenly and tended to raise the viability of the eggs, possibly due to the smaller temperature changes to which the eggs were exposed. Using this procedure, the eggs hatched at 21 to 22 days of incubation and intermittant opening of several of the eggs showed that they were at the correct level of development for their particular incubation period. It is for this last reason that the assumption of a zero hour age for the eggs as they entered the incubator was made.

CHAPTER II

EQUIPMENT AND INSTRUMENTATION

Ultrasonic Source

The ultrasonic source supplied by Hoffrel Instruments of Norwalk, Connecticut was a Model 310A 2.0 MHz transducer powered by a Model 101A Ultrasonoscope. It was designed for use as a brain midline echo check or abdominal scanner and had an output capability of both A and B modes. This unit was designed for the commercial market and models like it are in common usage today. The only alteration to the equipment was that the display phase had been modified so that only the strongest return signals would be shown on the cathode ray display tube. Since this alteration did not affect in any way the output of the transducer, the unit was considered for the purposes of these experiments to be typical of such commercially available units. In all experimentation, a constant voltage source powered the ultrasonic units so that the output of the ultrasonoscope did not vary with fluctuations in line voltage.

In order to further ascertain the character of the pulsed ultrasound, several experiments were performed to measure relevant parameters. These experiments and their results follow.

Average Intensity

The strength of the output of the transducer was measured using a radiation pressure balance based on Hill's design [32]. Figure 1 shows a representation of this balance. The balance used a brass plate

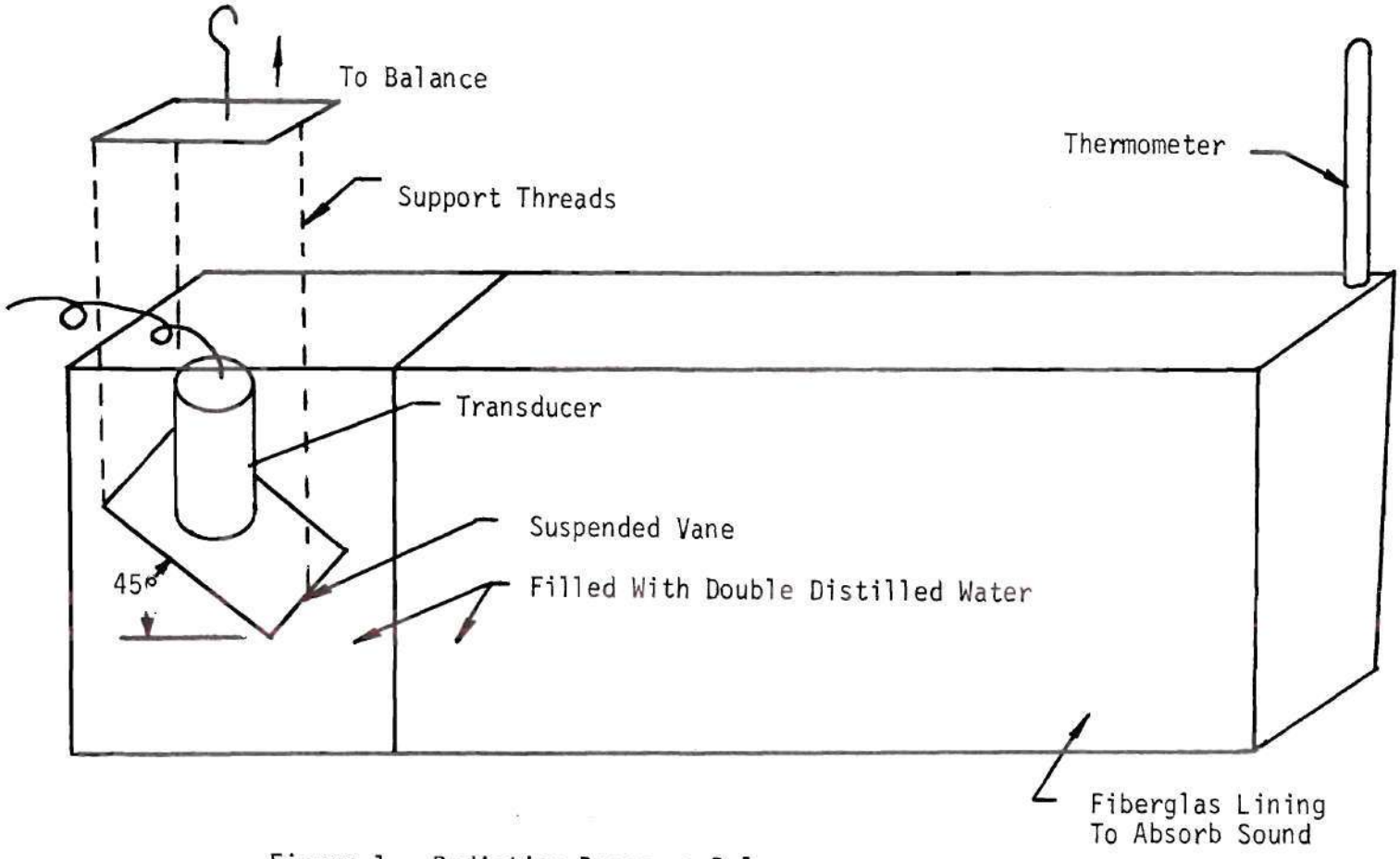

Figure 1. Radiation Pressure Balance

suspended from a very sensitive balance into a tank of degassed distilled water. The transducer was held in the water above the target facing down toward it so that when the transducer was activated, the target plate would experience a slight downward force, measured as fractions of a gram on the balance.

If the target plate was suspended horizontally below the transducer, an estimate of the amount of sound reflected and the amount absorbed would have had to been made since, if the signal were totally reflected, the power relationship would have been

$$W = \overline{F} \cdot 2\,\overline{V} \qquad (1)$$

(where W = power, $\overline{F}$ = force on target and $\overline{V}$ was the velocity of sound in water). But the power for totally absorbed sound was

$$W = \overline{F} \cdot \overline{V} \qquad (2)$$

since the former had twice the momentum change of the latter.

To eliminate this problem, the target plate was suspended at a 45° angle from level. In this way, any reflected signal was transmitted away from the system at right angles to the plane measured by the balance; thus, the system acted as if the target plate were perfectly absorbing. The sound energy reflected horizontally in this way was absorbed in fiberglas so that once the waves left the target area, they did not return to distort the measured signal. The speed of sound at 20°C in degassed distilled water is 1485 $m/_s$ + 2.4 ΔT [33]. Therefore, using equation (2) a correction factor was obtained for changing the balance readings to power readings. It was found that 67.5 mg. were measured for every watt of power output at 25°C. The Hoffrel ultrasonic

unit power output was controlled by a knob labeled "pulse power" which had eleven discrete settings from one to eleven. A set of ten readings were taken at settings three, four, seven and eleven in distilled degassed water at 25°C. The average of each of these ten readings was divided by the correction factor already obtained to calculate the total transducer time average power at each of these settings. Since the width of the transducer beam was one-half inch, these power output values were each divided by 1.27 cm^2 to obtain the time-space average intensities plotted in Figure 2. It should be noted that readings obtained for a given pulse power setting were very close to one another, thereby demonstrating the reproducibility of the system. This is consistant with Hill's balance data which showed a precision of $\pm$ 0.3 mW (the variance he found for ten measurements). However, if the entire balance setup was taken apart and then reassembled, a different average value was obtained. This disassembly and reassembly was necessitated by the lack of continuous access to the Mettler balance. The different average value was believed caused by having to aim the transducer by hand, and was the price to be paid for a portable system. Ten readings were made from ten different setups to assess this error. Care was taken to misalign the transducer as much or more than would have reasonably been expected in a given calibration study. The results demonstrated that the greatest error calculated as the difference between the highest and lowest reading divided by the lowest reading was 18%. This error was split into two parts, representing the error that the value read was too low, and the error that the value read was too high.

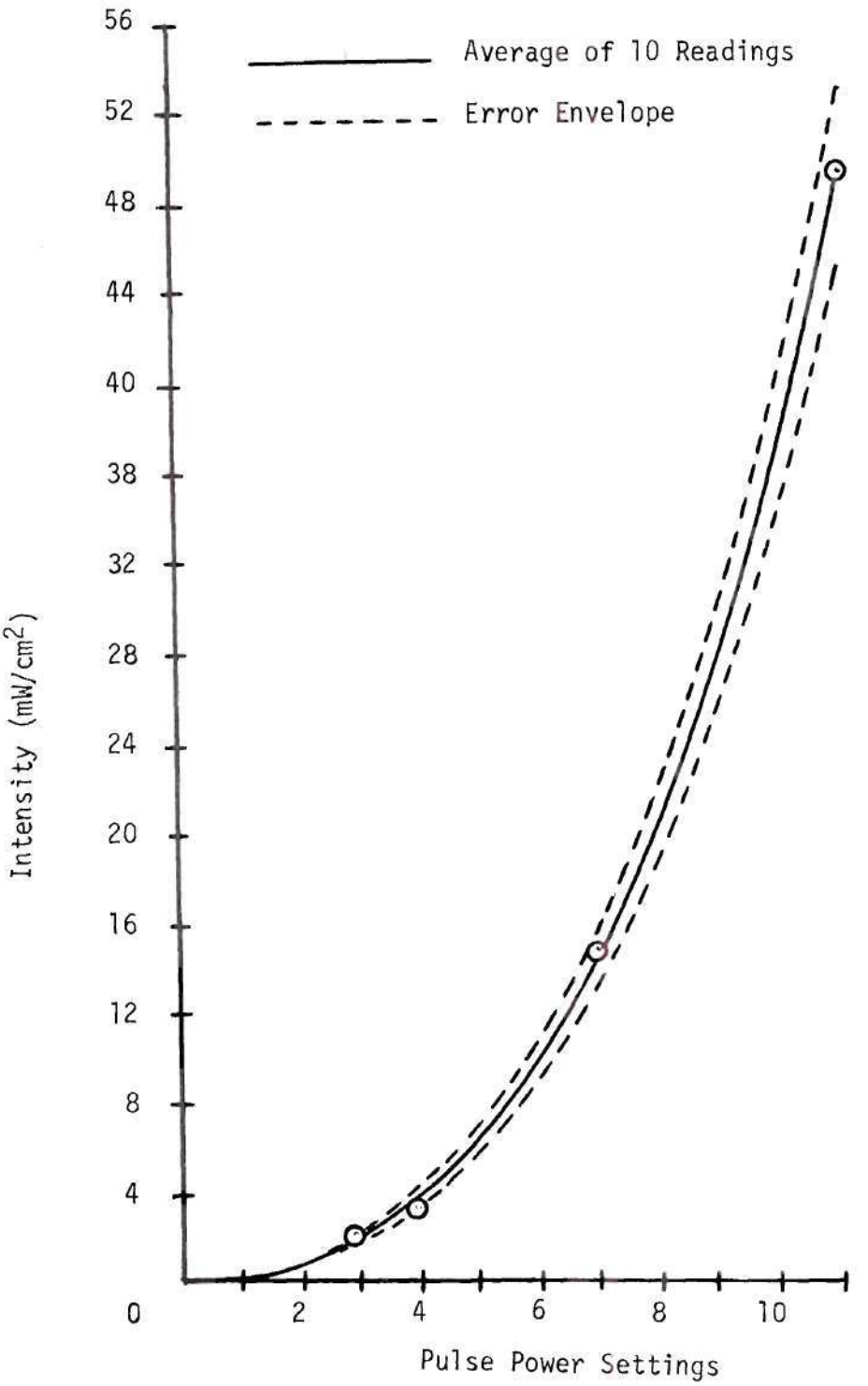

Figure 2. Plot of Intensity vs. Pulse Power Settings

These two curves (minimum and maximum) are plotted in Figure 2 and represent an error envelope.

These calibrations were made both before and after the insonation of the fertile eggs. No detectable change was found between these readings. Therefore, it was concluded that the intensities shown were those leaving the transducer during insonation.

Frequency Computation

The transducer was supposedly delivering ultrasonic pulses at a frequency of 2.0 MHz. However, this figure was found to be calculated by the manufacturer based on the thickness of the piezoelectric crystal used as an ultrasonic source. There is a direct relationship between the thickness of this crystal wafer and its free-free natural frequency; that is, its natural frequency with no boundary restrictions. Therefore, to manufacture a 2.0 MHz transducer, the thickness of the wafer is calculated, the wafer is cut and the transducer is stamped with the theoretical frequency of 2.0 MHz.

Obvious errors exist in this procedure. Even discounting the natural variations that must have existed in the piezoelectric crystal material, a much larger error is inherent in this procedure. To create pulsed ultrasound, a voltage is applied across the piezoelectric material for an instant and then turned off. This charge of energy causes the crystal to vibrate at its natural frequency as its output decays. The decay of the signal is caused by the damping material the piezoelectric crystal is packed with in the transducer. This damping material has the side effect of causing boundary restrictions on the

crystal, thus altering its natural frequency. Figure 3 shows a typical pulsed ultrasonic signal. Note that the frequency of the input pulses of voltage is several magnitudes lower than the vibrating frequency of the transducer. In order to calculate the actual frequency of the transducer's output, the ultrasonoscope was connected to a Tektronix Type RM35A oscilloscope as shown in Figure 4. The output shown on the cathode ray display tube of the oscilloscope was very similar to Figure 3. The abscissa of this plot was calibrated in units of time. Therefore, to calculate the natural or ringing frequency of the transducer, all that is needed is to divide the number of peaks of the ringing signal by the time these peaks encompass. This was found to be 1.45 MHz.

Repetition Rate

The repetition rate of the ultrasonic unit is the number of input voltage bursts per second. This was determined using the same method as the ringing frequency. This particular unit had a repetition rate of 454 Hz.

Stability

In order to determine the constancy of the output of the transducer, a study was done by photographing the "ringing" input signal to the transducer on an oscilloscope. Photographs of the scope display were made over several days, both when the ultrasound source had just been turned on as well as several random times afterward. A comparison of these photos revealed that there was no change in the input signal that could be visually detected. It was assumed that the output of the

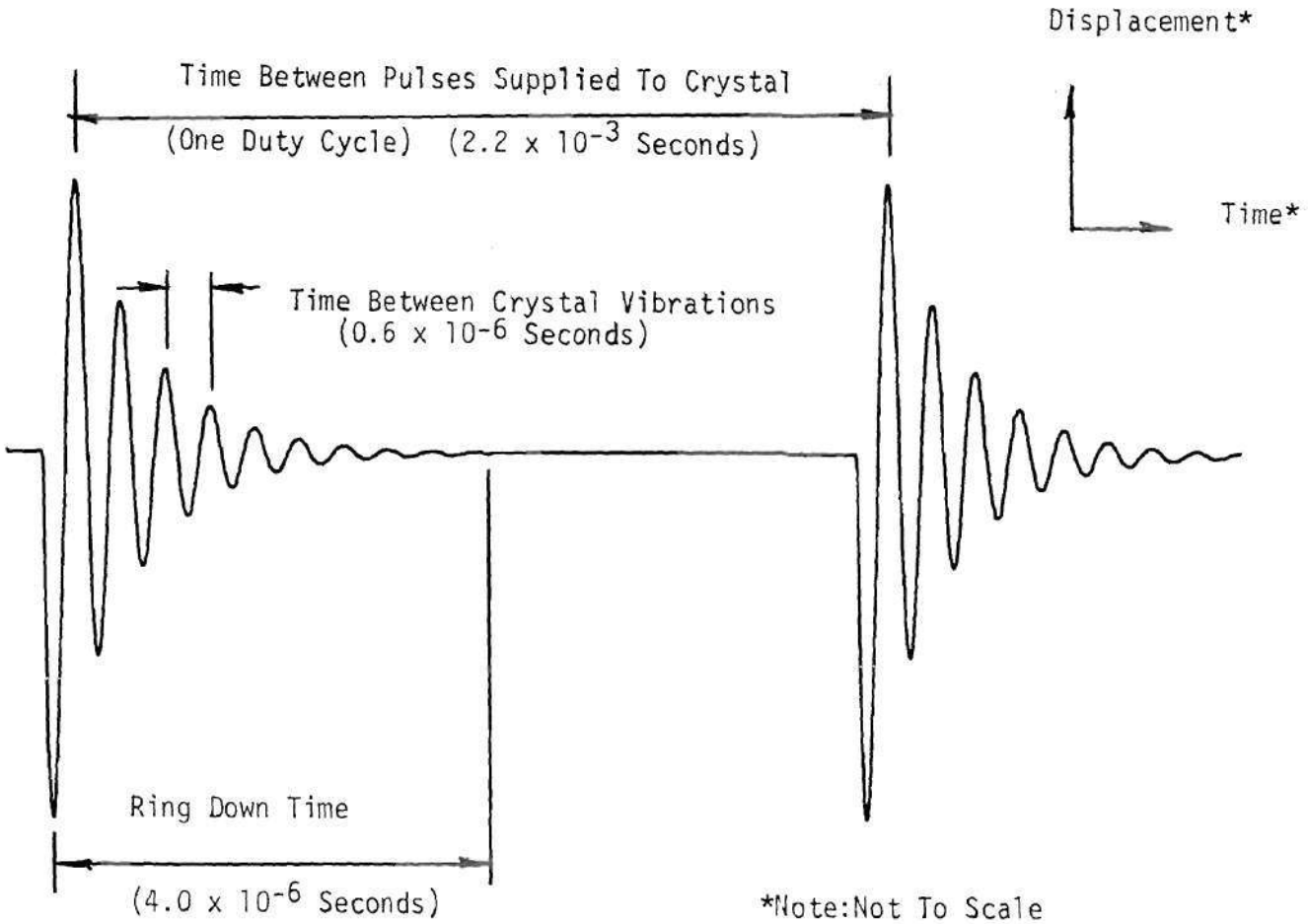

Figure 3. Typical Ultrasonic Signal

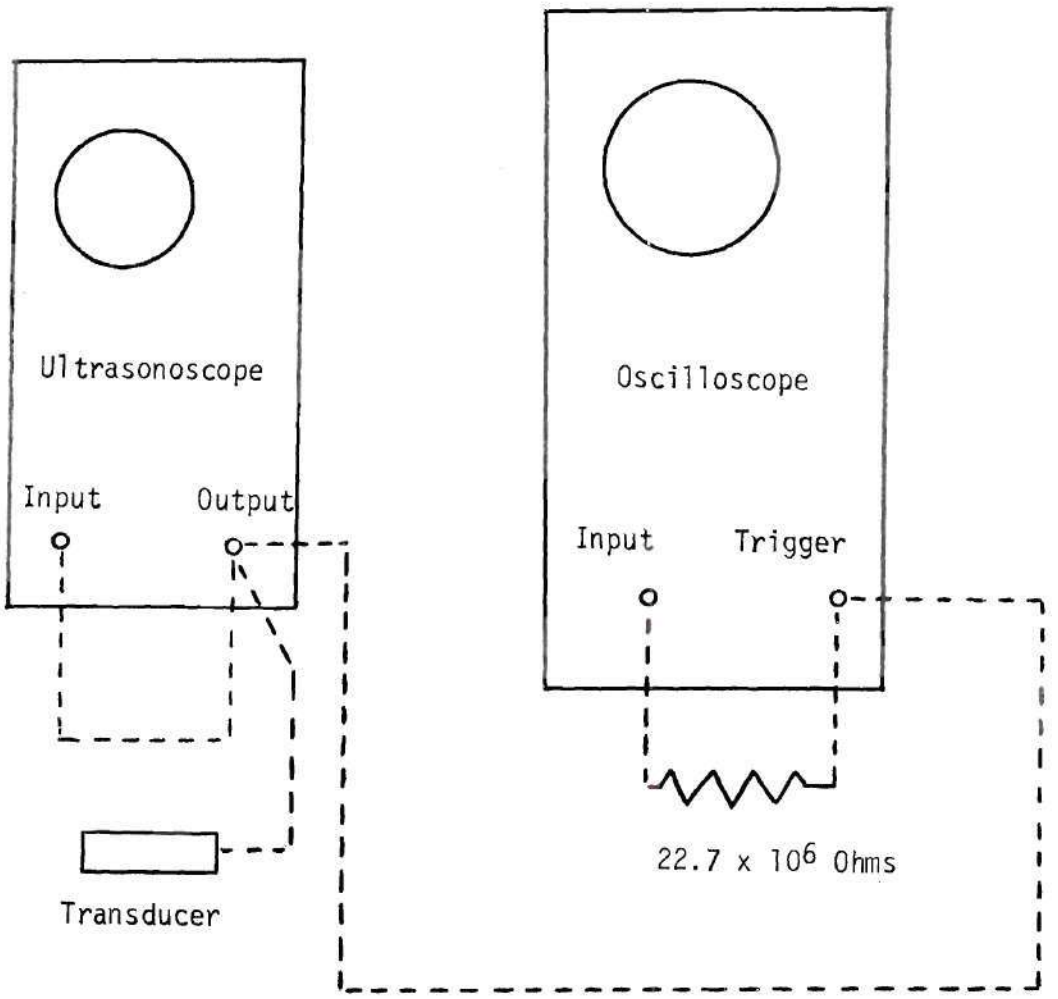

Figure 4. Schematic Diagram for Frequency Determination

transducer was stable given that the input signal was stable.

Miscellaneous Equipment

Several different units of instrumentation were employed during the procedures described in Chapters III and IV. The following is a list of this equipment and instrumentation. Remarks concerning the use of these instruments are given where pertinent.

Balance

The balance used to measure the time-space average intensities described earlier in this chapter was a Mettler Model H20. This unit contains a weighing platform which was removed during the intensity measurements. It was replaced with a steel plate containing a central hook to suspend it from the same point from which the weighing platform had hung. The brass target plate was suspended from this steel plate. Steel was used to make the dead weight of the new target system approximately the weight of the weighing platform. Only in this way could the balance be "zeroed".

Freeze Dryer

The embryonic samples which later were biochemically assayed as described in Chapter IV were first freeze dried in a Thermovac Freeze Dryer.

Colorimeter

The biochemical assays described in Chapter IV were based on a comparison of the absorbances (optical densities) of light at various wavelengths. Unknown solutions were compared with standard ones. The unit, a Bausch and Lomb Spectronic 20, introduced a small error of its

own. It is one of the few instruments of its type that has round input vials containing the solutions to be read for optical density. Most units have rectangular vials which can only be placed in the unit for analysis one way. The optical density readings can be greatly changed by variations in the optical characteristics of the vial through which the interrogating light beam passes. These rectangular vials eliminate this problem significantly by forcing the interrogating light beam to pass through the same two points in the vial.

Although no significant error was introduced into the experimental system due to this problem, it should be noted that most other colorimeters available on the commercial market do not share this error. By choosing another type of colorimeter, when available, the frustration of not being able to produce identical consecutive readings while analyzing the same solution would be eliminated.

Centrifuge

The centrifuge used in the separation procedures described in Chapter IV was the Dynac model manufactured by Clay-Adams, Inc. This unit was made largely of plastic, was portable, small and very inexpensive compared to other centrifuges commonly found in biochemical laboratories. Its only drawback was that the procedures required all centrifuging to be done at a temperature of about 4°C. This was easily remedied by placing the entire unit in an existing cold room already at this temperature.

Incubator

The forced-draft incubator used in the experiments described in

Chapter III and IV was a Favorite model made by Leahy Manufacturing Company. This unit had a larger egg capacity than necessary and was relatively inexpensive. Although it was acceptable for this work, several drawbacks should be noted. The humidity was controlled by placing pans of water on the heating element at the bottom of the incubator. This caused some fluctuation in the humidity. In addition, the temperature control was a thermocouple that was set by hand after observing the temperature on a separate thermometer. The manufacturer claimed that once set, the temperature would not vary more than 0.5°C. in either direction. However, the internal temperature of the incubator appeared to be somewhat affected by the external temperature, that is, the temperature of the room. Therefore, although the incubator did not vary about its mean temperature more than stated by the manufacturer, the mean temperature experienced some small changes. Small changes in the incubator temperature mean caused the rates of development of the embryos to rise and fall accordingly. This was one of the reasons that all the control groups of chick embryos did not have the same level of development when measured by somite counting as described in Chapter III.

CHAPTER III

SOMITE EXPERIMENTS

A study was made to determine the effects of diagnostic levels of ultrasound on the rate of growth of developing White Leghorn chick embryos. The number of somite pairs was chosen as the growth parameter. These block-like cell masses of mesodermic tissue first appear when the embryo is between twenty-three and twenty-six hours old. The subsequent regular increase in number of these somites makes them the most reliable criterion for determining the stage of development of young chicks [34]. Although there is much variation in somite number for chicks incubated for a given period of time, there is little variation between embryos with the same number of somites. Figure 5 shows the relationship between hours of development and number of somites. It should be noted here that the somites are actually arranged in pairs and, although the word "pairs" is usually omitted, it is understood that a fourteen somite embryo is one with fourteen pairs of somites.

Two separate experiments are described. In the first, the embryos were insonated, that is, subjected to ultrasound, with the shell left intact. However, serious questions arise as to the effects of the shell on the ultrasonic wave propogation. Therefore, a second series of experiments was performed, similar to the first except that the embryos were irradiated through windows cut in the shell. The results of both these experiments are compared.

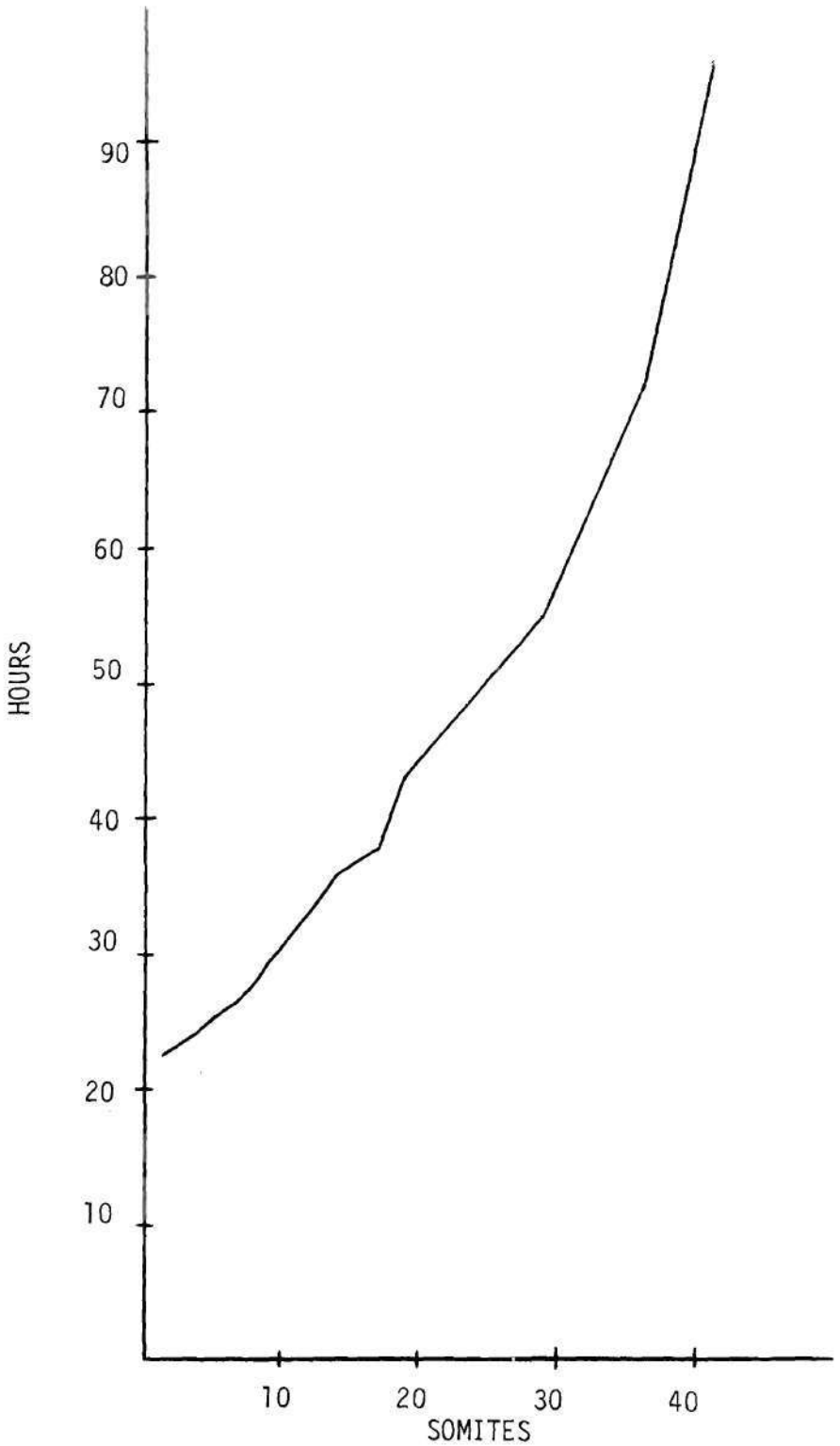

Figure 5. Relationship Between Hours of Development and Number of Somites [34]

First Somite Experiment

After fifty hours of incubation in the forced-draft incubator, the eggs were removed approximately a dozen at a time and placed on their side for several minutes. The shape of the egg is such that it has a blunt end and a pointed end. In cross-section the former is roughly circular while the latter is more elliptical. There is a point where these two contours "join" that represents the highest place on the side of the egg. By allowing the egg to rest on its side, the yolk, which is less dense than the surrounding albumin, floats to a position directly under this high point. Since the embryo formed on the surface of the yolk is more buoyant than the yolk, the embryo rests directly under this high point on the egg side (See Figure 6).

After several minutes of laying on their sides, the eggs were placed two at a time side by side in a gauze based holder. Care was taken to not rotate the eggs. The transducers were aimed straight down and placed against the shells of the eggs directly above the embryos. One transducer was a dummy, the other emitted ultrasonic pulses. A contact gel was used for coupling the transducers to the shells. In this way, any electromagnetic radiation from the "hot" transducer was exposed to both embryos approximately equally. Two intensities of ultrasound were used, approximately 15 or 49 mW/cm^2 as measured on the radiation pressure balance described in Chapter II. These correspond to pulse power settings of 7 and 11, respectively, on the ultrasonoscope. After insonation, the eggs were returned to the incubator for an addition-

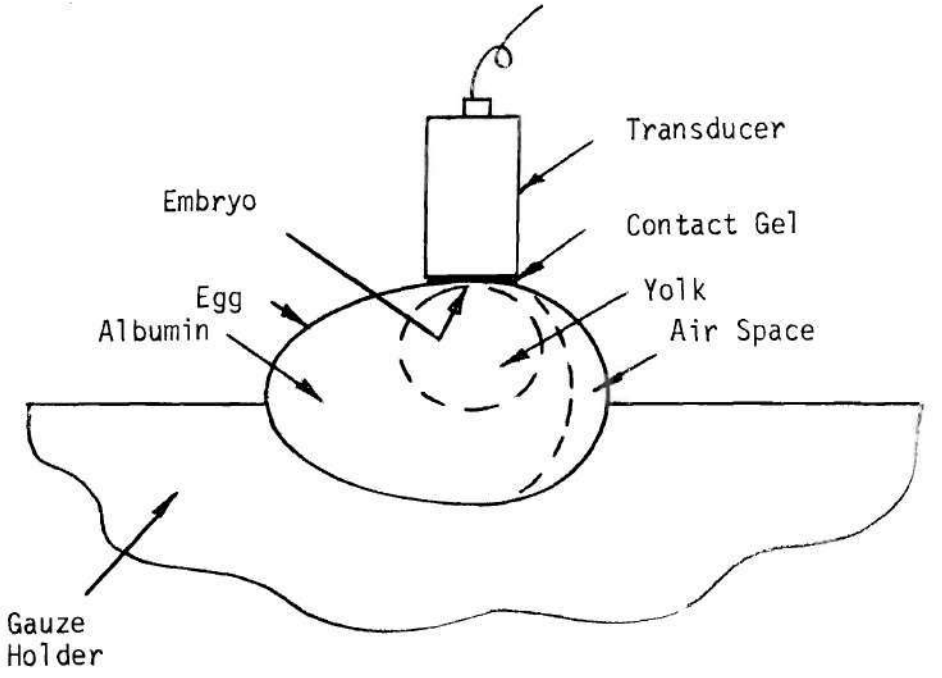

Figure 6. Insonation of Embryo

al fifty-two hours before opening.

When the embryos had been incubated for a total of 102 hours, they were removed from the incubator and opened into a large petri dish one at a time. Each embryo was separated from the yolk surface and placed in a second petri dish where it was washed with physiological saline (0.9% sodium chloride). The embryo was then moved to a dry petri dish and placed under a dissecting microscope. The amnion, an extra-embryonic membrane which encloses the embryo at this stage of development, was removed using dissecting needles. The somites were counted as the embryo lay on its side.

All eggs were insonated in random order, determined by sequentially assigning a number to each egg, then drawing eggs per a random number list. The eggs were opened in sequential order. In this way the somite count was not biased by previous knowledge of the ultrasound intensity given any particular egg. This experiment was performed six times.

The raw data is listed in Appendix A and a summary shown in Table 1. The statistical significance between each insonated group's mean and the corresponding control mean was based on the Student's "t" test [35]. A two-tailed test was used. All results of less than 80% were considered nonsignificant and listed as N.S. The sign convention was such that a positive "t" value indicated that the control mean was larger than the insonated mean. Although some groups of insonated embryos were shown to have a slight but statistically significant retarded level of development when compared to controls, other groups showed no signifi-

Table 1. Summary of Results of the First Somite Experiment

	(1) 15 mW/cm^2			(2) 49 mW/cm^2			(3) Controls		
Group	Mean	Sample Size	Standard Deviation	Mean	Sample Size	Standard Deviation	Mean	Sample Size	Standard Deviation
A	42.60	5	1.625	44.00	5	1.673	44.67	9	1.054
B	41.00	6	2.582	42.33	6	0.745	42.73	11	1.710
C	41.80	5	2.638	40.50	6	1.979	42.17	12	2.154
D	40.40	10	1.625	41.00	9	1.764	41.08	13	0.828
E	41.90	10	1.446	42.13	8	0.781	42.20	15	0.748
F	41.33	6	2.809	41.00	6	1.528	41.43	7	1.591

Group	Significance Between (1) & (2)	Significance Between (2) & (3)
A	.98	.84
B	.84	N.S.
C	N.S.	.84
D	N.S.	N.S.
E	N.S.	N.S.
F	N.S.	N.S.

cant difference at all. However, the means of even these latter groups were smaller than their controls. It must be remembered that although the Student's "t" test was designed for small sample sizes, the fluctuations in standard deviations caused by having relatively few samples could have large effects on the final statistical significance between two means. Therefore, the data were combined to enable a comparison to be made between means based on large sample sizes.

The initial impulse towards this end was to simply total all the data from the seven small groups, compute one mean and standard deviation for each intensity and perform the "t" test for significance between insonated and control levels of development. However, a serious problem would have arisen had this been done. It can be noted from Table 1 that the means of the control groups are not all the same. This was caused by variations in the temperature of the incubator as described in Chapter II. Also, the ratios of the sample sizes of insonated embryos to sample sizes of control embryos were not the same. Therefore, simple addition of all the somite values for statistical comparison would have caused an artificial addition to these differences between means, especially since it had already been determined that the control means were larger than the insonated ones. This problem would have been alleviated if the sample sizes of the insonated embryos equalled those of the controls. This was forced by throwing out all the "excess" control values in each group. For example, by referring to Appendix A it can be seen that five values made up the mean of the Group A somite counts at 15 mW/cm^2. Therefore, only the first five control values

of Group A were used to make up Table 2. In this way the sample sizes of each intensity somite total shown in Table 2 were equal and no artificial difference was created. The means and standard deviations for the totals are also shown in Table 2 as are the statistical significance between somite counts at each intensity and the control somite counts. The same sign convention explained previously was employed.

It can be seen from examining both Tables 1 and 2 that, although the statistical significance was small due to large variability, a clear trend is indicated. The trend being that for the six times this experiment was performed, all six results showed a slight decrease in the average number of somites of insonated chick embryos when compared with controls. A comparison of the 15 mW/cm^2 embryos with the 49 mW/cm^2 embryos showed no significant difference or trend.

Table 2

Modified Results of First Somite Experiment Using Equal Sample Sizes

	15 mW/cm^2		Control			49 mW/cm^2		Control		
Group	Σx_i	Σx_i^2	Σx_i	Σx_i^2	Sample Size	Σx_i	Σx_i^2	Σx_i	Σx_i^2	Sample Size
A	213	9087	222	9860	5	220	9694	222	9860	5
B	246	10126	260	11276	6	254	10756	260	11276	6
C	209	8771	209	8787	5	243	9865	251	10551	6
D	404	16348	408	16652	10	369	15157	368	15052	9
E	419	17577	422	17816	10	337	14201	338	14286	8
F	248	10298	247	10183	6	246	10100	247	10183	6
TOTAL	1739	72205	1768	74572	42	1669	69773	1686	71208	40

$\bar{x}$ = 41.40 $\bar{x}$ = 42.10 $\bar{x}$ = 41.73 $\bar{x}$ = 42.15

S = 2.194 S = 1.887 S = 1.830 S = 1.891

STATISTICAL SIGNIFICANCE OF DIFFERENCE IS .87

STATISTICAL SIGNIFICANCE OF DIFFERENCE IS N.S.

Second Somite Experiment

A second series of experiments was performed to ascertain the effect of insonating embryos through their shells. The incubation and insonation procedures were identical to those employed in the first somite experiments with one exception. After thirty-six hours of incubation, the fertile eggs were removed from the incubator and windowed. These 1 cm^2 windows were cut in the egg shells by using the edge of a cutting disk attached to a Dremel hand tool directly under where the transducer would later be placed. Care was taken not to injure the egg shell membrane which lies directly under the shell. The shell piece was removed with curved forceps. After some practice, it was found that about two-thirds of the eggs could be windowed without disturbing the membrane below. Only eggs which had been successfully windowed were placed back in the incubator. These windows provided a continuous soft tissue and liquid continuum between the transducer tip and the embryo. Note that only the 15 mW/cm^2 intensity was employed in this particular study.

As with the previous experiment, a contact gel was used to mate the transducer to the egg during insonation. There was no apparent effect on the embryos caused by placing the gel directly on the exposed membrane. In fact, after the eggs were returned to the incubator following insonation, most of the liquid content of the gel evaporated in the incubator heat and a vinyl-like layer of residue was left over the window. This residue affected the membrane by changing it from

opaque or translucent to clear. In this way, although it was not the purpose of these experiments, the embryo could have been observed at any subsequent time of development through a clear window in the shell, without having to open the egg or replace a section of the egg shell with a glass or plastic cup as is the common practice today.

The somites were counted as previously described in the first experiment and the results shown in Appendix B with a summary in Table 3. Since only one intensity was used, there were approximately the same number of insonated control embryos within each of the three groups. Therefore, the data was combined and a "t" test performed on the total number of insonated and control somites of all three groups. These totals and the statistical significance of a two-tailed test are shown in Table 3. The same sign convention used in the first somite experiment applies, i.e. positive significance indicates a control mean larger than the insonated mean.

Table 3 shows that for all three replications of this experiment, there was a slight but consistent increase in the average number of somites of the chick embryos when compared with the controls.

Table 3. Summary of the Results of the Second Somite Experiment

Group	Intensity							
	11.5 mW/cm^2			Control				
	Mean	Standard Deviation	Sample Size	Mean	Standard Deviation	Sample Size	"t" Value	Statistical Significance
A	43.00	2.236	4	42.40	1.840	5	-0.653	-N.S.
B	42.42	1.076	12	42.55	5.157	11	-0.410	-N.S.
C	44.00	1.091	11	43.55	.782	11	-2.641	-.98
TOTAL	43.15	1.483	27	42.93	1.719	27	-1.815	-.92

Interpretation of Results

The results of the first series of somite experiments clearly show a small but statistically significant decrease in the growth rate of the insonated embryos. However, when the same experiments were rerun insonating the embryos through windows in their shells, the exact opposite results were obtained. Several conclusions were drawn from these facts.

Both series of experiments were repeatable. The difference between windowed and nonwindowed results gave an indication of the effect the shell had on the ultrasound. Although no theory is presented as to the exact mechanism of action of the shell, the fact that a shell effect existed was obvious. It was also obvious that the embryos were definitely affected ty the ultrasound treatment with or without the window. This difference was the rationale for the further experimentation described in Chapter IV.

CHAPTER IV

BIOCHEMICAL ASSAY

Introduction

It was established in Chapter III that a small but reproducible change in the growth rate of chick embryos subjected to diagnostic levels of ultrasound could be detected. The change was slight enough that it probably produced no gross effect, which was in accord with the previous publications. However, what of the secondary effects? Would this shift in the gestation process produce adults with behavioral modifications or would the adult completely recover? Trauma at the embryological level causing modification in the behavior of the adult has been well documented [36].

No answer to these questions can be found from the previous somite experimentation. In fact, even the questions themselves are nothing more than speculation. There is an independence of development between the head and trunk regions of the embryo so that retardation of the trunk indicated by a slight decrease in somite count would not necessarily justify the assumption that this retardation also was occurring in the region of the embryo containing the brain. Consequently, a series of biochemical assays of several growth-indicating macromolecules of the brain were performed in order to determine if changes in these parameters between insonated and control embryos could be measured and also to determine if these changes were of more, less, or the same

significance as those changes in the trunk region. The three parameters analyzed were the ribonucleic acids (RNA), the deoxyribonucleic acids (DNA), and the total protein. The brain region of each embryo was assayed by separating the head from the trunk and removing the beak and eyes. What remained was mostly brain tissue and was designated as such. The body of the embryo had its heart and limbs removed before assay so that only changes in the trunk would be considered. The heart and limbs develop somewhat independently of the trunk and would only have confused the results.

Method of Procedure

Preparation of Embryos

The White Leghorn fertile eggs were incubated and insonated in exactly the same manner as described in the First Experiment section of Chapter IV with one exception. Instead of opening all the eggs at 102 hours (approximately 4 days), some of the eggs were allowed to continue developing in the incubator until they were eight or twelve days old (102 hours + 48 or 96 hours respectively).

The four day embryos were opened one at a time into a large petri dish. The embryo was separated from the yolk surface and placed in a second petri dish where it was washed with physiological saline (0.9% sodium chloride). Then the embryo was moved to a dry petri dish and placed under a dissecting microscope. The amnion was removed using dissecting needles. Also, the eyes and limb buds were removed, as was the heart. The head was then separated from the trunk by slicing

the embryo's neck laterally directly below the auditory vesicles. The head and trunk pieces were then placed in separate vials and immediately frozen at -10°C. Previous experience had shown that five heads or trunks were needed to get a satisfactory assay for 4 day embryos. Therefore, each vial of 4 day old embryological parts contained five heads or five tails. In addition, the parts were assigned to vials so that if five heads were in one vial, the trunks from these same five embryos were placed in a corresponding vial.

The 8 and 12 day embryos were prepared by opening the eggs one at a time into a large petri dish. The embryo was removed from its surrounding tissues with forceps. The embryo was transferred to another petri dish where it was washed with the physiological saline solution. Then the embryo was placed in a dry petri dish for dissection. No dissecting microscope was used since the 8 day embryos were large enough to work on with the naked eye. The head was separated from the body by slicing through the neck laterally at the neck's thinnest point. The eyes and beak were removed from the head. The heart and limbs were removed from the trunk. Then the heads and trunks were placed in separate vials so that there were three heads or trunks in each vial. As with the 4 day embryos, the parts were distributed so that if one vial contained three 8 day or 12 day heads, a corresponding vial contained the trunks of the same embryos. The vials were frozen at -10°C. It should be noted here that although one head or trunk from the 8 and 12 day embryos would have been sufficient tissue from which to obtain an acceptable biochemical assay, three heads or trunks were

combined. Since there was a great variability between the exact stage of development of these embryos, it was thought that assaying the combined tissue of several heads or trunks would lower this variability.

The frozen tissue was stored in a freezer until collection of all embryonic samples was complete. In all, ninety vials were processed, thirty 4 day, thirty 8 day, and thirty 12 day embryos shows the contents of each vial.

In order to simplify the assay procedure, all tissue was freeze dried for 24 hours at -40°C at a pressure of 150 microns of Mercury. The tissue was then ground into a fine powder with mortar and pestle over dry ice. Equal samples of 10 mg. were weighed on the Mettler balance from the 8 and 12 day embryo vials. The vials of 4 day embryos already contained approximately that measure. The samples were then ready for biochemical assay.

Although the basic methodology of the Schmidt-Thannhauser procedure of separation of RNA, DNA, and protein was used [37], so many modifications had to be made so that meaningful results could be obtained that the modified procedure is included in this text in its entirety. In this way, anyone wishing to duplicate the results would be better prepared to do so. The steps are listed chronologically and explain how to separate the 10 mg. tissue samples into three solutions containing RNA, DNA and protein respectively. In the processing of the ninety vials, all thirty 4 day embryos were separated at once, as were all thirty 8 day embryos and all thirty 12 day embryos. Makeup of all solutions are given in Appendix F.

Separation Procedure

1. Preheat the water bath to 37°C.

2. Defrost the samples.

3. Place the samples in ice bucket.

4. Weigh out 1.0 gm. of DOC (Deoxycholic acid) and 0.5 gm. of Brij 58 (Polyoxyethylene 20 Cetyl Ether).

5. Mix in a small beaker (20 ml.) 1.0 gm. of DOC and 9.0 ml. distilled water. (Makes 10% solution).

6. Mix in a small beaker (20 ml.) 0.5 gm. of Brij 58 and 9.5 ml. distilled water. (Makes 5% solution).

7. Place the 10.0 mg. sample of defrosted tissue in ground glass homogenizer tube with 4 ml. of .01M Tris pH 7.3 SDS buffer and homogenize.

8. Transfer this solution to a 12 ml. conical centrifuge tube.

9. Wash homogenizing tube with an additional 1 ml. of Tris and add this to centrifuge tube used in previous step.

10. Add 0.5 ml. of DOC solution and 0.5 ml. of Brij 58 solution to the homogenate.

11. Stir well with vortex and let the solution sit for 10 minutes in ice. This will solubilize the lipid.

12. Add 2 ml. of 20% TCA (trichloroacetic acid) to each tube and let them sit in ice for 20 minutes after gently stirring with glass rod. The precipitate formed contains RNA, DNA, and protein.

13. Centrifuge samples @2500 rpm. for 20 minutes (use swinging bucket centrifuge).

14. Pour away the supernatant and let the tubes drain upside down. Note; Be careful not to let pelletized precipitate slide out of the tube.

15. Add 2 ml. of 0.5N NaOH to each tube and vortex.

16. Heat the tubes in the water bath @37°C. for 2 hours. This hydrolizes the RNA.

17. Remove the tubes and reset the water bath to 90°C.

18. Add 3N HCl dropwise to neutralize the solution. Use litmus paper to check the pH and record the number of drops added to each tube. It should take 6-10 drops.

19. Add an equal volume of 20% TCA to each tube (2 ml. + drops).

20. Gently stir with a glass rod and let the tubes sit in ice for 20 minutes.

21. Spin at 2500 rpm. for 20 minutes in a swinging bucket centrifuge.

22. Pour the supernatant (which contains the RNA in solution) into test tubes and set them aside in a refrigerator.

23. Drain the centrifuge tubes upside down being careful not to lose the pellet which contains the DNA and protein.

24. Add 2 ml. of 10% PCA (Perchloric acid). This solubilizes the DNA.

25. Stir with a glass rod to loosen the pellet and return the tubes to the ice.

26. Put the centrifuge tubes in the 90°C. water bath for 20 minutes.

27. Remove the tubes from the bath and place them in ice for 20 minutes.

28. Spin @2500 rpm. for 20 minutes in the swinging bucket centrifuge.

29. Pour the supernatant containing DNA into test tubes and set them aside in the refrigerator.

30. The pellet remaining in the bottom of the centrifuge is the protein from the original tissue sample.

There should be three test tubes of material generated by this procedure for every sample of tissue processed, one test tube with the protein pellet, one test tube with the RNA in 4 ml. of a 10% TCA solution, and one test tube containing the DNA in 2 ml. of 10% PCA.

Several notes of caution on the above procedure should be mentioned. If violent mixing such as vortexing is used after the TCA or PCA is added, some of the precipitate formed will adhere to the sides of the test tube causing a loss of material to the pellet formed since the centrifuge cannot pelletize solid material not suspended in the solution. In addition, this solid material on the tube sides will come out when the supernatant is poured off, thereby contaminating the supernatant and causing loss of material to the pellet. Also, if fixed bucket rotors are used in the centrifuge instead of swinging bucket rotors, instead of getting a compact pellet formed in the bottom of the tube, the precipitate will form a smear down the lower side. This smear is not as compact as the pellet from a swinging bucket and, therefore, large amounts of precipitate which should remain in the

tube will come off with the supernatant.

DNA Assay

The DNA was assayed by the diphenylamine reaction [38]. This reaction used diphenylamine in the DNA solution as a color indicator with acetaldehyde as a catalyst to the reaction. DNA was indicated by a light bluish color. The more DNA in solution, the deeper the blue. The Bausch and Lomb Spectronic 20 colorimeter described in Chapter II was used to measure the percent of absorbance of light (optical density) at a wave length of 595 millimicrometers. A reading was also made of each sample at 700 millimicrometers to get a background contamination reading. The final optical density reading was the difference of the two. In the range of DNA concentration encountered in this experiment, there was a linear relationship between the amount of DNA in solution and the optical density (O.D.). To demonstrate this linearity, a standard curve was created by processing five samples each of five different concentrations of DNA. Appendix D shows the data and calculations for the standard curve, which is shown in Figure 7.

The exact procedure used for determining the DNA assay follows:

1. Preheat the water bath to 30°C.
2. Place a 0.1 ml. pipette in freezer.
3. Make five standards of 0.5 ml. DNA (100 micrograms/ml. H_2O) + 0.5 ml. distilled water + 1.0 ml. 20% PCA.
4. Make one blank of 1.0 ml. distilled water and 1.0 ml. 20% PCA.
5. Mix a 4% solution of diphenylamine in glacial acetic acid

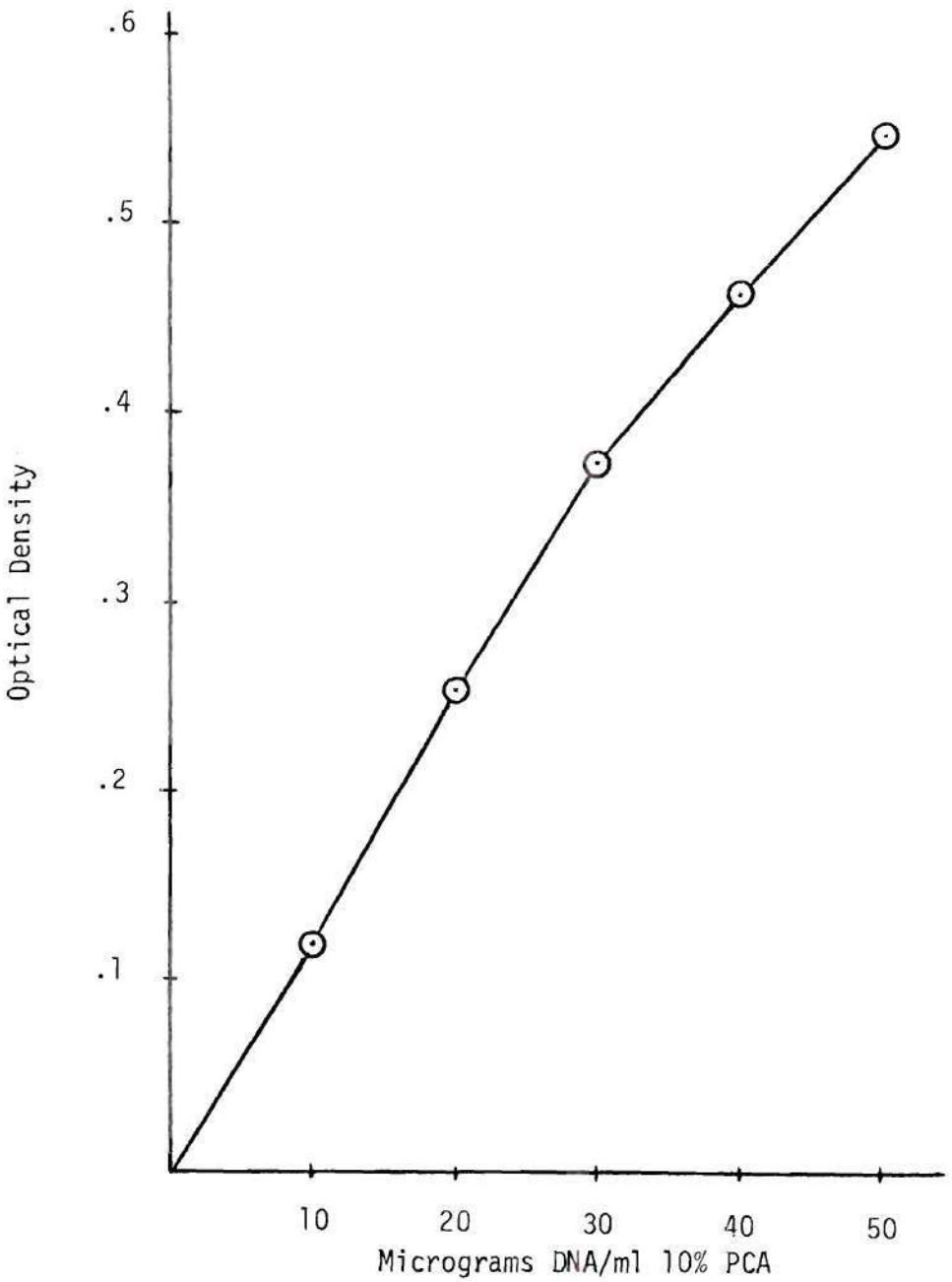

Figure 7. DNA Standard Curve

(2.0 gm. diphenylamine brought up to 50 ml. in glacial acetic acid). This solution is unstable and must be made fresh each time.

6. Add 2.0 ml. of 4% solution of diphenylamine to each test tube and vortex.

7. Add 0.1 ml. of the acetaldehyde solution (1.6 mg/ml) to each test tube. Use the frozen pipette and keep the acetaldehyde on ice since this solution boils at room temperature.

8. Mix solutions well on vortex.

9. Cover the test tubes with parafilm and place them in the 30°C. water bath overnight.

10. Read the optical density of each test tube at 595 millimicrometers. After all readings are made, repeat the readings and average. Do the same for 700 millimicrometers. The difference between the averages of the optical densities at 595 and 700 millimicrometers is the final O.D. used. See Appendix E for typical method of converting optical density to micrograms of DNA.

RNA Assay

The RNA assay was based on the orcinol reaction [39]. This reaction used an orcinol reagent as a color indicator just as the DNA was indicated by diphenylamine. The proportion of RNA in the solution was indicated by the intensity of green in the unknown solution. Although the unknown solutions to be assayed contained RNA in 4 ml. of a 10% TCA solution, only 0.2 ml. of this solution was processed since using all the unknown would result in a green tinted sample too dark to be read by the colorimeter. As with the DNA assay, all vials including

standards were read for O.D. on the colorimeter, then read again and the two values were averaged. The optical density was read at 660 millimicrometers. No background reading was needed since the orcinol reaction is not as sensitive to contamination as was the diphenylamine reaction.

A standard curve was prepared from thirty readings of standards, five standards at each of six different concentrations of RNA. The data from which the standard curve was plotted appears in Appendix D and the curve is shown in Figure 8. Note that once again the concentrations of RNA were linearly proportional to the optical densities for this range of concentrations. See Appendix E for typical method of converting optical density to micrograms of RNA.

The procedure used for determining the RNA assay follows:

1. Boil water with boiling beads over burner.

2. Prepare enough orcinol reagent so 2.0 ml. of reagent will be available for each sample including five standards and one blank. This solution oxidizes rapidly and must be prepared fresh each time. The orcinol reagent is made by bringing 1.0 gm. of recrystallized orcinol up to 100 ml. with cupric ion reagent. See Appendix F for the formula of cupric ion reagent.

3. Make one blank of 2 ml. of 10% TCA.

4. Make five standards of 0.4 ml. of 100 micrograms RNA/ml. distilled water + 0.6 ml. distilled water + 1.0 ml. 20% TCA. This solution gives a concentration of 20 micrograms RNA/ml. 10% TCA.

5. Prepare the unknown test tubes with 0.2 ml. of the unknown

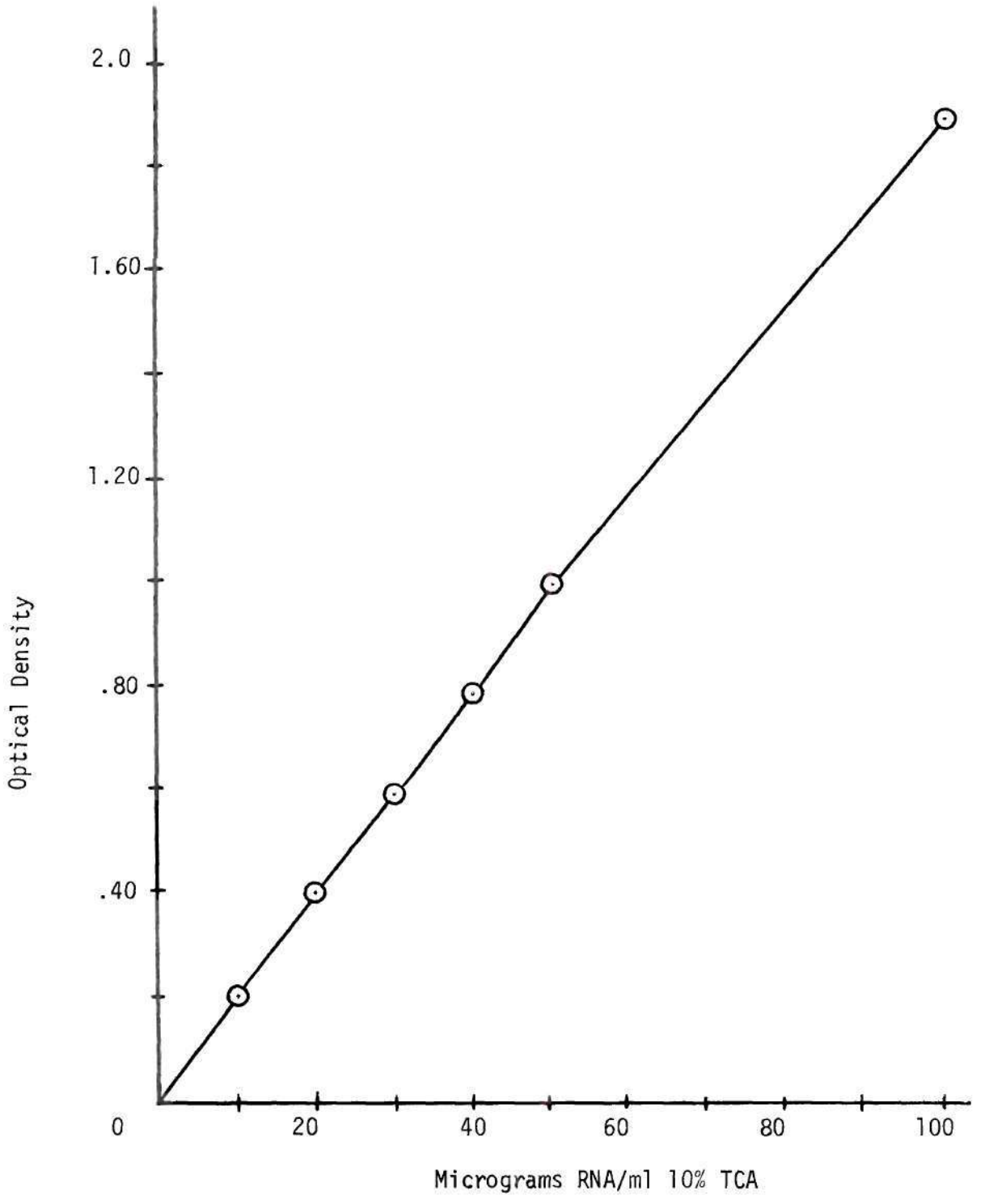

Figure 8. RNA Standard Curve

solution.

6. Add 2.0 ml. of the orcinol reagent to each test tube.

7. Place a marble on top of each test tube.

8. Place the test tubes immediately in boiling water.

9. Allow them to remain in boiling water for 45 minutes. This is sufficient time for the greenish color to develop fully.

10. Remove the test tubes from the boiling water, remove the marbles and chill the test tubes in ice for 30 minutes.

11. Read the O.D. of each tube at a wavelength of 660 millimicrometers.

12. Repeat the previous step and average the two values for each test tube.

Protein Assay

The protein assay was similar to that of RNA and DNA in that a colorimetric analysis was employed. BSA (bovine serum albumin) was used as a standard for the analysis. Originally, the Lowry method [40, 41] was to be used to determine the protein assay. However, while doing trial runs, it became evident that more protein was contained in the pellets than the Lowry procedure could accommodate. Therefore, the Biuret reaction [42] was selected since this reaction was not only able to indicate the large quantities of protein, but it was also more accurate since it only indicated peptide bonds while the Lowry reaction read peptide bonds and phenol groups as well, which would have confused the results.

The Biuret procedure is as follows:

1. To each test tube with a protein pellet, add 1.0 ml. of 0.5N NaOH. This will solubilize the protein and bring it into solution. (1.2 ml. approximately).

2. Place these test tubes in the water bath at 37°C. for two hours, stirring intermittently with a glass rod.

3. Prepare five standards of 0.6 ml. 10 mg. BSA/ml. H_2O + 0.6 ml. of 1N NaOH. (This is a 5 mg. BSA/ml. 0.5N NaOH solution.)

4. Prepare a blank of 1.2 ml. 0.5N NaOH.

5. To all test tubes add 4 ml. of Biuret reagent.

6. Allow the solution to stand at room temperature for thirty minutes.

7. Read the O.D. at 550 millimicrometers.

8. Repeat step 7 and average the values.

Typical calculations for converting O.D. to milligrams of protein are shown in Appendix E. The linearity of the relationship between O.D. and concentration of BSA (which is all protein for practical purposes) is demonstrated by calculation of a standard curve. Five readings were made at each of five concentrations of BSA in 0.5N NaOH. The data are shown in Appendix D and the curve is plotted in Figure 9.

Experimental Results

The total amounts of RNA, DNA, and protein assayed for each sample are shown in Appendix G. It should be reiterated that the results give the total amount of protein and DNA in each 10 mg. sample of tissues, but

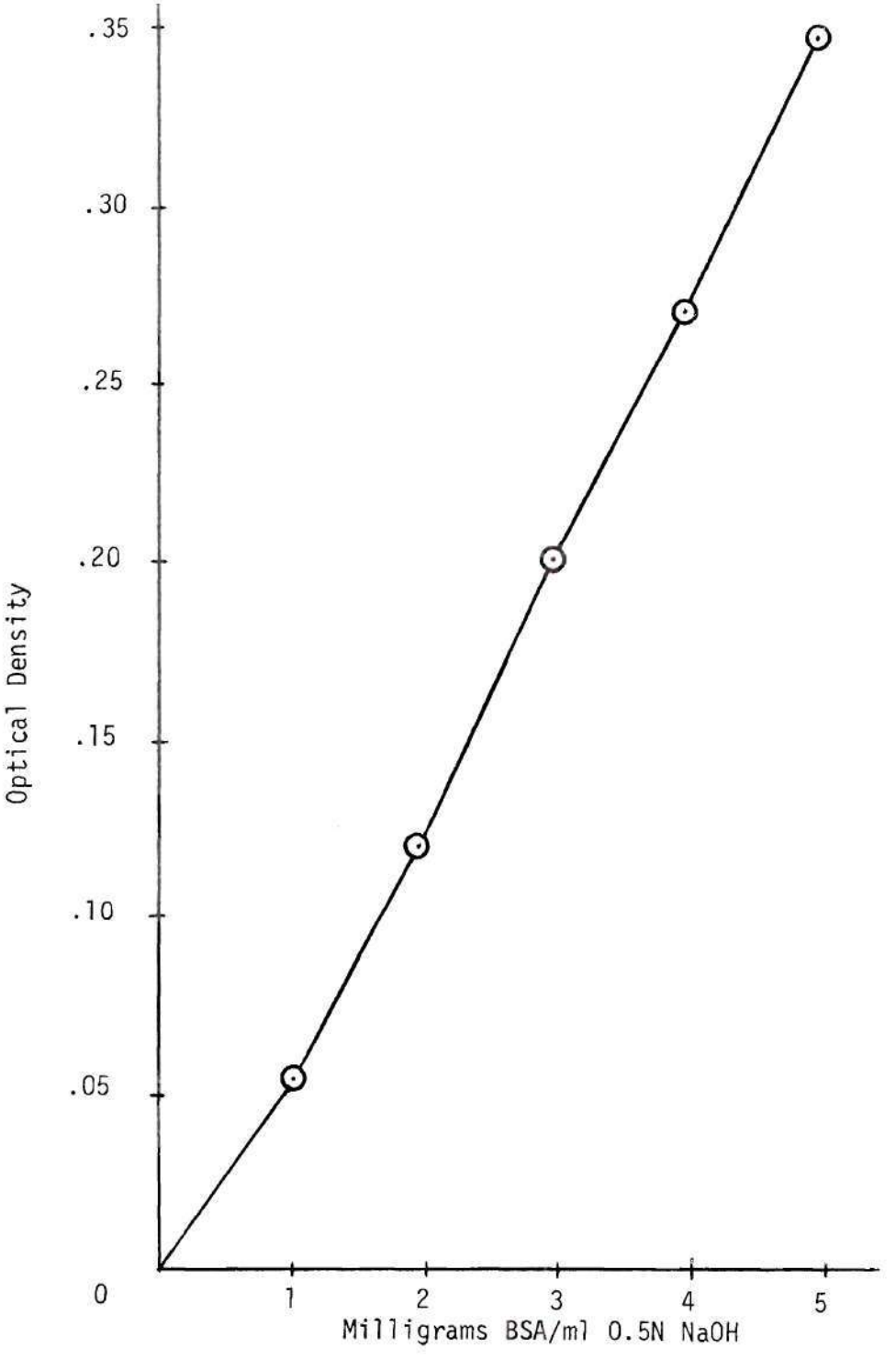

Figure 9. Protein (BSA) Standard Curve

that the RNA values shown are only one-twentieth of the total RNA in each sample since only 0.2 ml. of the 4.0 ml. of unknown from each vial was assayed. Since the analysis of these results was based on comparison of means rather than on absolute numbers, these results as listed are perfectly suitable for statistical analysis. For interpretation purposes, these values of RNA, DNA, and protein were presented as ratios in Appendix G. The ratios were micrograms of RNA per microgram of DNA and milligrams of protein per milligram of DNA. Since there is the same amount of DNA in every nucleus of every cell in each embryo, this is the same as expressing the results as micrograms of RNA per cell and milligrams of protein per cell. Note that "milligrams of protein per cell" measures the average amount of total protein for every cell in the embryo, which is not the same as the amount of protein in each cell since much of the protein is extracellular. Appendix H shows the condensation of these ratios expressed as group mean, standard deviation, and sample size. The Student's 't' test was used to determine the statistical significance between each group of embryonic parts for a given age, intensity and part type [35]. This statistical test compares the group means and using a weighted standard deviation based on both variances calculates the intervals of confidence that the two means statistically differ. The results of these calculations are shown in Tables 4, 5, and 6. A two-tailed test was used since there was no reason to presume that the insonated tissue would have larger or smaller values as compared with the controls. The sign convention of the values of "t" was that a positive "t" indicated the insonated

Table 4. Statistical Results of Four Day Embryos

Insonated Group		Control Group		P/D* or R/D*	Intensity**	Head or Trunk	"t" Value	Degrees of Freedom	Statistical Significance In Means
Group Number	Group Mean	Group Number	Group Mean						
A	.0454	E	.0503	P/D	1	Head	.847	7	N.S.
C	.0588	E	.0503	P/D	2	Head	-1.573	8	-.84
B	.0526	F	.0475	P/D	1	Trunk	-.896	8	-N.S.
D	.0466	F	.0475	P/D	2	Trunk	.393	8	N.S.
A	.182	E	.245	R/D	1	Head	3.801	7	.99
C	.265	E	.245	R/D	2	Head	-2.397	8	-.93
B	.296	F	.227	R/D	1	Trunk	-1.926	8	-.90
D	.244	F	.227	R/D	2	Trunk	-1.401	8	-.80

* P/D = comparison is of the means of milligrams of protein per micrograms of DNA.

R/D = comparison is of the means of micrograms of RNA per micrograms of DNA.

** 1 = 15 mW/cm^2 2 = 49 mW/cm^2

Table 5. Statistical Results of Eight Day Embryos

Insonated Group		Control Group		P/D*		Head		Degrees	Statistical
Group Number	Group Mean	Group Number	Group Mean	or R/D	Intensity**	or Trunk	"t" Value	of Freedom	Significance In Means
G	.0566	K	.0768	P/D	1	Head	2.235	8	.92
I	.0657	K	.0768	P/D	2	Head	1.291	8	N.S.
H	.0874	L	.0752	P/D	1	Trunk	-.682	8	-N.S.
J	.0941	L	.0752	P/D	2	Trunk	-1.709	8	-.86
G	.183	K	.230	R/D	1	Head	1.254	8	N.S.
I	.209	K	.230	R/D	2	Head	.479	8	N.S.
H	.281	L	.253	R/D	1	Trunk	-.457	8	-N.S.
J	.315	L	.253	R/D	2	Trunk	-1.267	8	-N.S.

* P/D = comparison is of the means of milligrams of protein per micrograms of DNA.

R/D = comparison is of the means of micrograms of RNA per micrograms of DNA.

** 1 = 15 mW/cm^2 2 = 49 mW/cm^2

Table 6. Statistical Results of Twelve Day Embryos

Insonated Group		Control Group		P/D* or R/D*	Intensity**	Head or Trunk	"t" Value	Degrees of Freedom	Statistical Significance In Means
Group Number	Group Mean	Group Number	Group Mean						
M	.0607	Q	.0770	P/D	1	Head	7.166	8	.99
O	.0684	Q	.0770	P/D	2	Head	2.340	8	.93
N	.0624	R	.0706	P/D	1	Trunk	1.605	8	.84
P	.0705	R	.0706	P/D	2	Trunk	.020	8	N.S.
M	.195	Q	.231	R/D	1	Head	2.269	8	.93
O	.229	Q	.231	R/D	2	Head	.125	8	N.S.
N	.212	R	.228	R/D	1	Trunk	1.241	8	N.S.
P	.237	R	.228	R/D	2	Trunk	-.488	8	-N.S.

* P/D = comparison is of the means of milligrams of protein per micrograms of DNA.

R/D = comparison is of the means of micrograms of RNA per micrograms of DNA.

** 1 = 15 mW/cm^2 2 = 49 mW/cm^2

mean was smaller than the control, while a negative "t" showed the insonated mean to be larger than that of the control. Any statistical significance below 80% was considered to be nonsignificant and was listed as N.S. Figures 10, 11, 12 and 13 show plots of the ratios of RNA/DNA and protein/DNA for both heads and trunks. The numbers by each data point represent the probability that these points differ from the controls.

Interpretation of Results

Although this biochemical assay produced information concerning the amount of RNA, DNA, and protein in both heads and trunks of insonated and control embryos, the data are meaningless unless certain biological processes are taken into consideration when the data are analyzed.

Protein may be thought of as a long chain of amino acids attached to each other in a long line, end to end, like the cars of a train. These amino acids are linked together by peptide bonds. The Biuret reaction used to measure the amount of protein in a given sample was actually measuring the number of these peptide bonds. Since any two protein chains of the same length have about the same number of bonds, the Biuret reaction results give a good indication of the total amount of protein in a given tissue sample. The information dictating the order of amino acids for any given type of protein to be manufactured is contained in the DNA of the cell in its nucleus. The amino acids which will eventually be linked to form protein chains are initially separate and free from one another in the cell's cytoplasm, where the protein

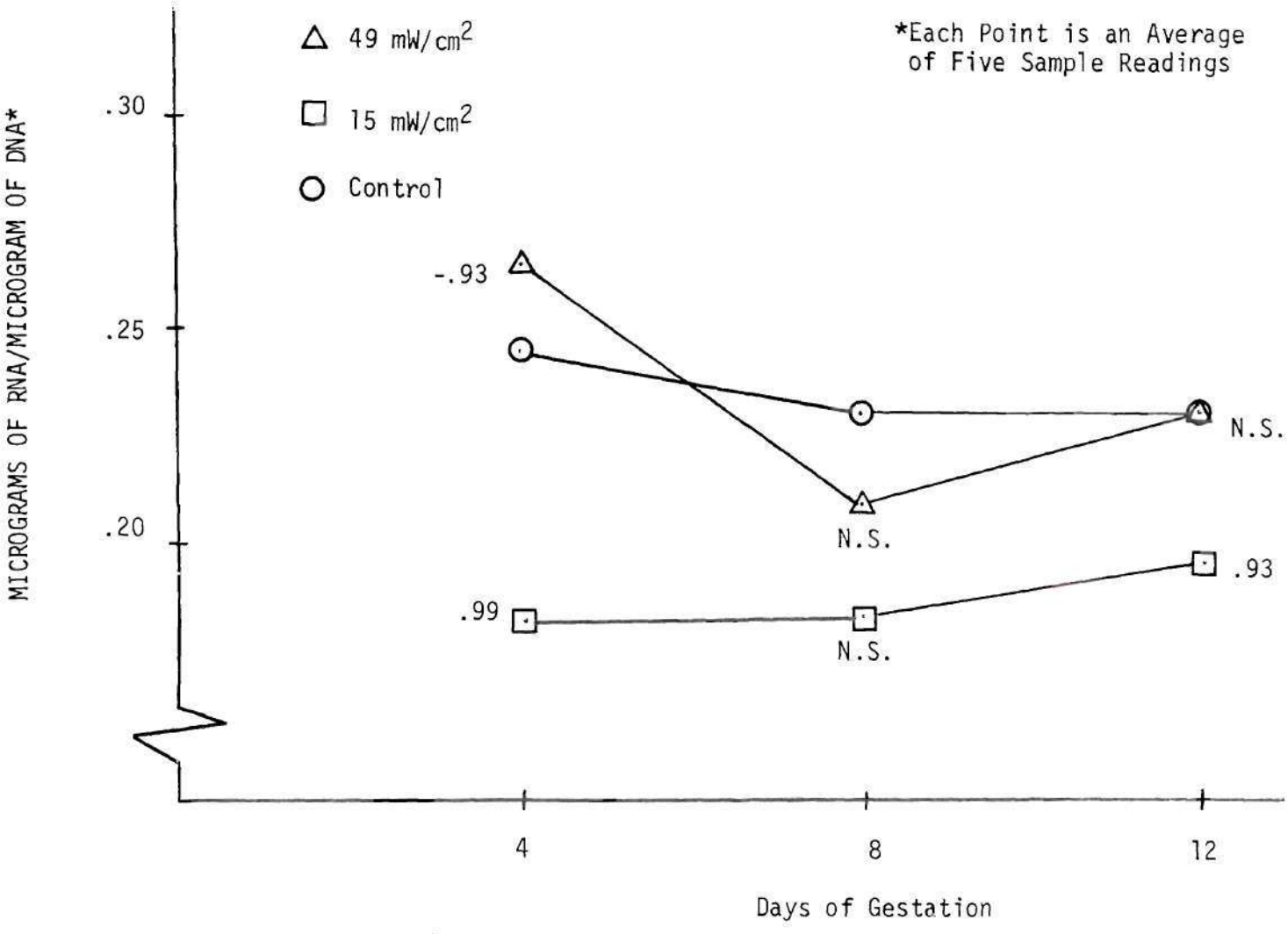

Figure 10. Micrograms of RNA per Microgram of DNA of Embryonic Heads

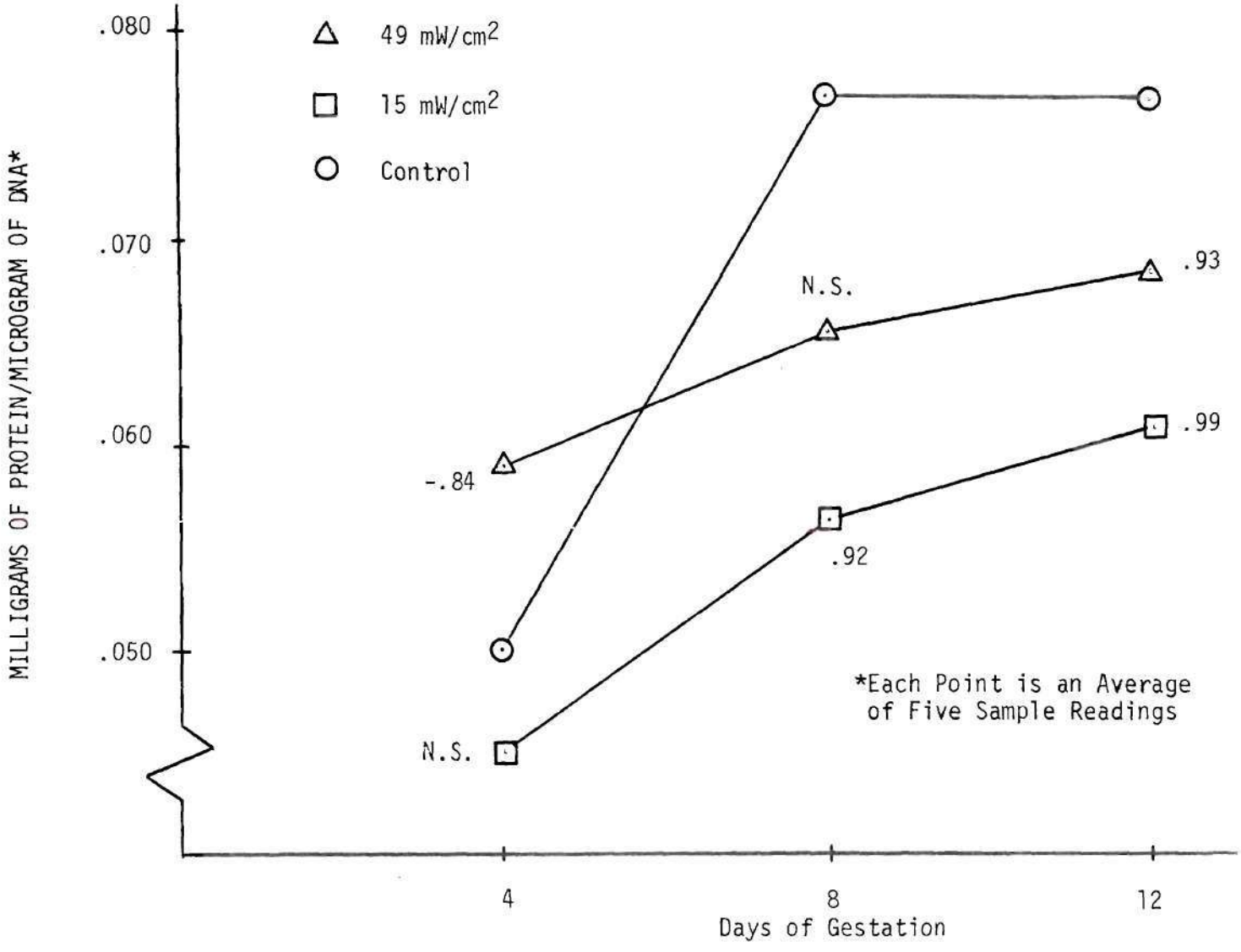

Figure 11. Milligrams of Protein per Microgram of DNA of Embryonic Heads

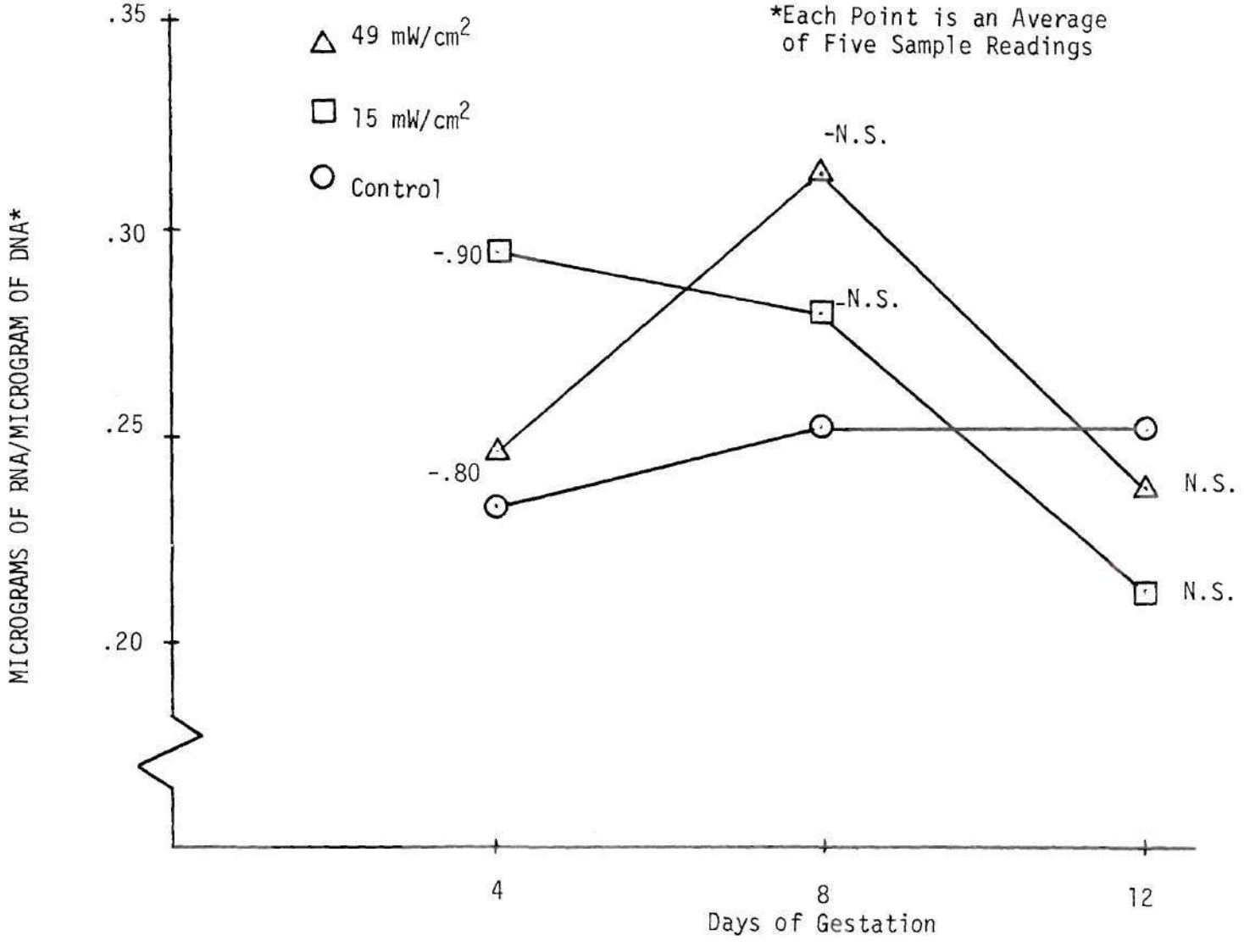

Figure 12. Micrograms of RNA per Microgram of DNA for Embryonic Trunks

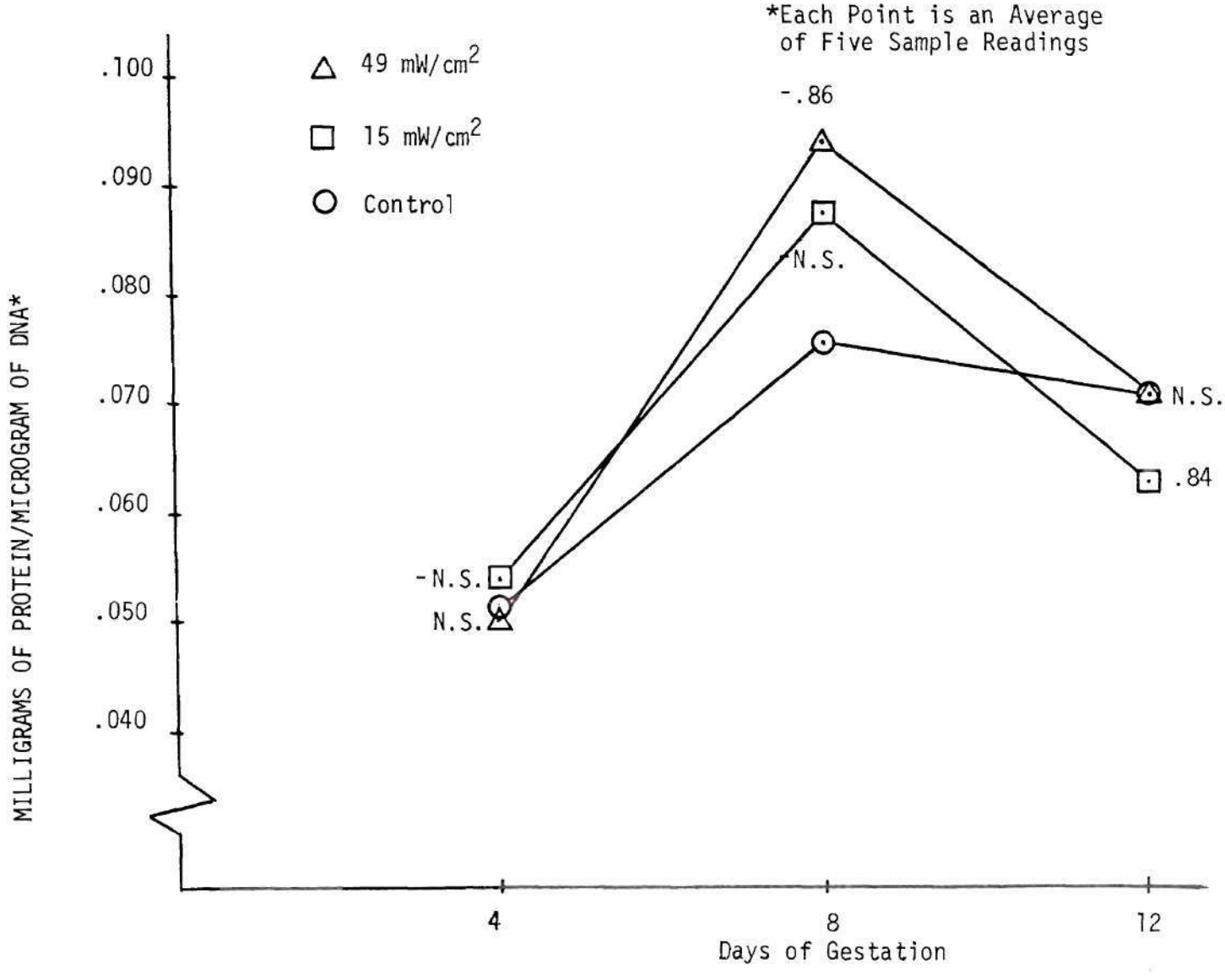

Figure 13. Milligrams of Protein per Microgram of DNA for Embryonic Trunks

chain will eventually be manufactured. It is the RNA which copies the message of amino acid order from the DNA, takes this information to the cytoplasm, and helps form the chain of amino acids in the proper order. Therefore, there is a definite inter-relationship between the RNA, DNA, and protein.

The amount of DNA in every cell remains constant. However, the amount of RNA can vary greatly, as can the amount of protein. When more protein is to be made, more RNA is first manufactured to facilitate the protein synthesis. There is evidence that in some systems of the embryo, the rise in RNA content occurs several days before the rise in protein synthesis [43]. However, protein can and does undergo degradation. The shrinking of the size of the embryonic tail as the embryo develops and the formation of finger and toe digits in the limb buds by degradation of the protein between the digits are examples of this. Since the protein assay only measured the total amount of protein at each stage of development, no determination can be made as to whether changes in protein levels were caused by changes in the rate of synthesis or changes in the rate of degradation or both.

However, by analyzing the graphs in Figures 10 through 13, certain trends do indeed become evident. These graphs also show the statistical significance as well as the value difference between the insonated and control embryonic parts. It would be well to remember that although the Student's "t" test was designed for small sample sizes, the fluctuations in variances caused by having relatively few samples can have large effects on the final statistical significance

between two mean values. However, by analyzing trends in the plots while comparing statistical significance, the effect of ultrasound on the developing embryo can be postulated.

It can be seen from Figure 10 that at four days of development, there is a significant deficit in the amount of RNA of brain insonated at 15 mW/cm^2. This trend continues through both the eight and twelve day readings and is again significant statistically at twelve days. However, the trend at eight days is such that, although the "t" test does not show it, the difference is still there. Figure 11 shows that this deficit in RNA is associated with an increasing significant deficit in protein which by twelve days is highly significant. This would seem reasonable based on the previous discussion of the time inter-relationship between RNA and protein. Although the brain is insonated at 49 mW/cm^2 show a higher level of RNA at four days when compared with the controls, there is a higher level of protein indicated at this time also. By eight days, the amount of RNA in these brains has decreased, and the level of protein has decreased also. By twelve days the level of protein is significantly decreased as was the case when the lower intensity was used.

Figures 12 and 13 show more mixed results. The effect of ultrasound in the trunk region seems to have been an increase in the RNA of the insonated chicks causing a significant increase in protein content by eight days and dropping thereafter. These increases and decreases of protein seem to correspond to increases and decreases of RNA. However, none of the differences in RNA or protein in the trunk

region have high statistical significance, and it may well be that these assay procedures were not sufficient to define these differences more accurately. Another possibility is that the degradation rate of protein in the trunk region was affected. No definite conclusions seem to be warranted, therefore, based on the trunk data as they exist. This is not to say, however, that the definite trends and statistical significances of the head region are any less meaningful. The independence of the development between head and trunk regions make it very possible that the ultrasound could have had a greater effect on the developing brains than on the developing trunks of these embryos.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

In Chapters III and IV it was shown that a statistically small but consistent decrease in the growth rate of the chick embryos was induced by subjecting these embryos to diagnostic levels of ultrasound for three minutes. Biochemical assays of the brain regions showed that the ultrasound had caused a decrease in the total amount of RNA, which was later manifested as a decrease in the total amount of protein. Although these differences in RNA and protein did not demonstrate consistent statistical significances due to the wide variations of the samples involved, the trends shown in Figures 10 and 11 clearly indicate the possibility of ultrasonically induced deviations from the norm.

A two-fold approach is proposed to investigate the effect of ultrasonically induced protein deficiency in the developing brain, (1) the effects on the embryonic chick brain should be further investigated and (2) possible behavioral modifications of adult rats resulting from treatment with ultrasound during embryogenesis should be studied.

To determine the effects of ultrasound on the macromolecular content of embryonic chick brains, an isotope labeling study is proposed. Labeled uridine, a nucleic acid specifically used in the formation of RNA, would be injected into eggs at the end of 48 hours of incubation. The fertile eggs would then be subjected to diagnostic levels of pulsed ultrasound and allowed to develop further. At various ages, some of the

embryos would be removed, the RNA extracted, and its radioactivity determined by liquid scintillation counting. The amount of radioactivity is a measurement of RNA synthesis. Comparisons with controls would determine the effect of ultrasound on new RNA production. This same basic technique could be used to determine the amount of new protein synthesized, except that an isotopic labeled amino acid mixture should be used instead of uridine. The results of this research, when compared with the investigator's previously mentioned data, should pin down the effect of diagnostic levels of ultrasound have on the RNA and protein content of the chick brains, especially the question of recovery.

To investigate the question of behavioral modification, rats could be mated and pregnant females insonated at different times during gestation. After birth, the progeny would be subjected to various types of intelligence testing, such as learning and unlearning simple tricks, unlearning times, etc. The results would be analyzed and conclusions drawn. These experiments are of utmost importance in that the effects would be tested on a mammalian system very similar to our own. The previous experimentation had used chick embryos which, while they are very convenient for embryological work, the effect the shell has on the ultrasonic propagation is undoubtedly much different than the effect of the soft tissues in the mammal. If the biochemical shifts described above are peculiar to avian systems due to the particular interaction between sonic wave and shell, then no cause for alarm exists. However, the preliminary results show that the possibility of behavioral modification exists, and study of this possibility in a mammalian system soon is certainly indicated.

Besides the research described above, additional study should be directed toward investigating calibration techniques, especially concerning possible transducer focusing, so that this very low intensity non-ionizing pulsed radiation can be monitored in a clinical setting without access to a major ultrasonic research center. The transducers on the commercial market today are calibrated at most one time after manufacture and then placed in unlimited clinical use without some periodic check. The safety of this practice is in doubt because of the present uncertainty about the biological effect of ultrasound.

APPENDIX A

SOMITE COUNTS OF FIRST SOMITE EXPERIMENT

INTENSITY (mW/cm^2)	
	GROUP A
15	44, 41, 45, 41, 42
49	44, 42, 44, 43, 47
Control	44, 46, 44, 44, 44, 43, 45, 46, 46
	GROUP B
15	41, 46, 40, 42, 38, 39
49	42, 42, 44, 42, 42, 42
Control	45, 41, 43, 43, 44, 44, 39, 42, 42, 45, 42
	GROUP C
15	37, 41, 43, 44, 44
49	39, 40, 40, 38, 44, 42
Control	41, 42, 47, 42, 37, 42, 43, 42, 43, 43, 41 43
	GROUP D
15	42, 38, 39, 38, 42, 41, 39, 42, 41, 42
49	43, 41, 43, 40, 40, 43, 38, 42, 39
Control	42, 40, 41, 41, 41, 40, 41, 42, 40, 40, 42, 42, 42

INTENSITY (mW/cm^2)	
	GROUP E
15	43, 44, 42, 42, 40, 41, 43, 42, 39
49	41, 43, 42, 43, 41, 43, 42, 42
Control	43, 43, 41, 42, 42, 41, 43, 43, 41, 43, 43, 42, 42, 42, 42
	GROUP F
15	43, 38, 44, 44, 37, 42
49	39, 39, 42, 43, 42, 41
Control	42, 41, 39, 41, 40, 44, 43

APPENDIX B

SOMITE COUNTS OF SECOND SOMITE EXPERIMENT

INTENSITY (mW/cm^2)	GROUP A
15	44, 42, 40, 46
Control	45, 42, 42, 42, 41
	GROUP B
15	43, 42, 42, 42, 43, 43, 41, 42, 42, 45, 43, 41
Control	39, 46, 40, 44, 42, 44, 39, 42, 44, 43, 45
	GROUP C
15	45, 44, 43, 45, 45, 43, 44, 45, 45, 43, 42
Control	42, 44, 43, 44, 45, 43, 44, 43, 44, 44, 43

APPENDIX C

CONTENTS OF VIALS #1-90

Vial Numbers	Assay Group	Heads (H) Trunks (T)	Numbers of Embryos	(mW/cm^2) Ultrasonic Intensity	Age of Embryos (days)
1 - 5	A	H	5	15	4
6 - 10	B	T	5	15	4
11 - 15	C	H	5	49	4
16 - 20	D	T	5	49	4
21 - 25	E	H	5	Control	4
26 - 30	F	T	5	Control	4
31 - 35	G	H	3	15	8
36 - 40	H	T	3	15	8
41 - 45	I	H	3	49	8
46 - 50	J	T	3	49	8
51 - 55	K	H	3	Control	8
56 - 60	L	T	3	Control	8
61 - 65	M	H	3	15	12
66 - 70	N	T	3	15	12
71 - 75	O	H	3	49	12
76 - 80	P	T	3	49	12
81 - 85	Q	H	3	Control	12
86 - 90	R	T	3	Control	12

APPENDIX D

DATA FOR STANDARD CURVE

Data for Calculation of DNA Standard Curve

(1) O.D. @595 Millimicrometers	(2) O.D. @700 Millimicrometers	(1)-(2) Net O.D.
Group A (50 micrograms DNA/ml 10% PCA)		
.54	.00	.54
.54	.00	.54
.56	.00	.56
.54	.00	.54
.56	.00	.56
Group B (40 micrograms DNA/ml 10% PCA)		
.45	.00	.45
.46	.00	.46
.49	.00	.49
.46	.00	.46
.47	.01	.46
Group C (30 micrograms DNA/ml 10% PCA)		
.39	.00	.39
.39	.01	.38
.36	.00	.36
.38	.00	.38
.38	.00	.38
Group D (20 micrograms DNA/ml 10% PCA)		
.25	.00	.25
.25	.00	.25
.27	.00	.27
.27	.00	.27
.24	.00	.24
Group E (10 micrograms DNA/ml 10% PCA)		
.14	.00	.14
.11	.00	.11
.12	.00	.12
.12	.00	.12
.11	.00	.11

Data for Calculation of RNA Standard Curve

Optical Density at 660 Millimicrometers

Group A (100 micrograms RNA/ml 10% TCA)

1.9
1.9
1.9
1.9
1.9

Group B (50 micrograms RNA/ml 10% TCA)

1.0
1.0
1.0
1.1
1.1

Group C (40 micrograms RNA/ml 10% TCA)

.80
.79
.77
.80
.80

Group D (30 micrograms RNA/ml 10% TCA)

.60
.54
.65
.47*
.60

Group E (20 micrograms RNA/ml 10% TCA)

.40
.43
.35
.42
.41

*Rejected, not included in calculations

Group F (10 micrograms RNA/ml 10% TCA)

.20
.21
.22
.19
.20

Data for Calculation of Protein Standard Curve

Optical Density at 550 Millimicrometers

Group A (5 milligrams BSA/ml 0.5N NaOH)

.35
.34
.33
.35
.35

Group B (4 milligrams BSA/ml 0.5N NaOH)

.27
.27
.25
.27
.27

Group C (3 milligrams BSA/ml 0.5N NaOH)

.18
.20
.20
.21
.20

Group D (2 milligrams BSA/ml 0.5N NaOH)

.12
.15
.12
.12
.12

Group E (1 milligram BSA/ml 0.5N NaOH)

.05
.06
.05
.06
.05

APPENDIX E

TYPICAL METHOD OF CONVERTING OPTICAL DENSITY TO MICROGRAMS OF DNA

(Although the following method was specifically for DNA in Appendix D, the same method was used to determine conversion factors for RNA and protein.)

Each group contains readings of optical densities (O.D.) from five different samples of the same concentration. To calculate a conversion factor for converting O.D. to micrograms of DNA, each standard group's average was determined by dropping the highest and lowest readings and averaging the middle three. This gave five distinct values of O.D., one for each of the five concentrations. These points were plotted in Figure 7 and a "best fit" line was projected through them. Observe that there is a very linear relationship between O.D. and amount of DNA in this range of concentrations.

When a series of unknown concentrations was analyzed, a set of five identical standards was analyzed with them. The highest and lowest readings of the standards were dropped, and the middle three readings averaged. Since linearity had been established by Figure 7, the ratio of concentration of DNA in the standards to average O.D. constituted a conversion factor, so that when this conversion factor was multiplied by the O.D. readings for

the experimental run in question, the result was given in units of micrograms of DNA.

APPENDIX F

SOLUTIONS

1. .01M. Tris pH 7.3 0.1% SDS buffer

Bring 1.21 gm. Tris and 1 gm. SDS (sodium dodecyl sulfate) up to 700 ml. with distilled water. Titrate with concentrated HCl on pH meter until 7.3 reading is achieved. Bring up to 1 liter with distilled water. This solution is stable but must be kept in a refrigerator. Warm before using to dissolve the SDS.

2. Orcinol reagent

Dissolve 1.0 gm. of recrystallized orcinol in 100 ml. of cupric ion reagent. This solution is unstable and must be made fresh each time.

3. Cupric ion reagent

Dissolve 0.15 gm. of $CuCl_2 \cdot H_2O$ in 100 ml. of concentrated HCl. Solution is very stable. If $CuCl_2 \cdot 5H_2O$ is used, add 0.19 gm.

4. Biuret reagent

Dissolve 1.50 gm. $CuSO_4 \cdot 5H_2O$ and 6.0 gm. sodium potassium tartrate in 500 ml. distilled H_2O. Add with constant stirring 300 ml. of 10% NaOH. Dilute to 1 liter with distilled H_2O. This solution is stable, but discard if red or black precipitate forms with time.

APPENDIX G

BIOCHEMICAL ASSAY DATA

4 DAY EMBRYOS

Vial No.	(1) Micrograms of RNA	(2) Micrograms of DNA	(3) Milligrams of Protein	RNA/ DNA	Protein/ DNA
1	*				
2	9.78	42.5	1.64	.230	.0386
3	6.94	48.3	1.85	.144	.0383
4	9.50	55.2	2.28	.172	.0413
5	7.12	39.3	2.49	.181	.0634
6	9.02	40.3	1.64	.224	.0407
7	14.54	41.4	2.80	.351	.0676
8	12.36	30.9	1.92	.400	.0621
9	12.82	50.4	2.24	.254	.0444
10	9.30	36.9	1.77	.252	.0480
11	12.36	42.5	1.98	.291	.0466
12	12.64	47.5	2.55	.266	.0537
13	8.08	31.6	2.41	.256	.0763
14	14.06	55.7	3.59	.252	.0645
15	14.54	56.4	2.97	.258	.0527
16	11.34	44.0	2.05	.258	.0466

*Sample Lost

4 DAY EMBRYOS

Vial No.	(1) Micrograms of RNA	(2) Micrograms of DNA	(3) Milligrams of Protein	RNA/ DNA	Protein DNA
17	10.73	41.9	1.98	.256	.0473
18	10.08	38.8	1.84	.260	.0474
19	10.58	48.8	2.15	.217	.0441
20	9.47	41.4	1.98	.229	.0478
21	12.85	51.4	2.63	.250	.0512
22	11.84	47.7	2.29	.248	.0480
23	12.49	54.9	2.95	.228	.0537
24	13.85	56.3	2.98	.246	.0529
25	11.99	47.0	2.15	.255	.0457
26	8.06	32.0	1.39	.252	.0434
27	8.82	40.9	1.77	.216	.0433
28	8.82	43.2	2.08	.204	.0481
29	10.23	43.1	2.01	.237	.0466
30	8.46	37.8	2.12	.224	.0561

8 DAY EMBRYOS

Vial No.	(1) Micrograms of RNA	(2) Micrograms of DNA	(3) Milligrams of Protein	RNA/ DNA	Protein/ DNA
1	8.86	73.1	2.92	.121	.0399
2	8.16	34.6	2.42	.236	.0699
3	9.33	80.0	2.82	.117	.0353
4	9.33	44.0	2.99	.210	.0673
5	9.10	39.5	2.78	.230	.0704
6	11.80	57.8	3.13	.204	.0542
7	9.47	28.1	3.25	.337	.1157
8	10.87	55.3	3.39	.197	.0613
9	11.43	24.7	3.39	.463	.1372
10	10.17	49.4	3.39	.206	.0686
11	8.30	86.4	3.39	.096	.0392
12	8.77	52.9	3.46	.166	.0654
13	9.00	30.6	2.46	.294	.0804
14	8.54	34.6	2.71	.247	.0783
15	8.63	35.5	2.32	.243	.0654
16	10.50	25.7	2.78	.409	.1082
17	10.64	27.2	3.11	.391	.1143
18	8.30	36.0	2.96	.231	.0822
19	10.50	34.6	3.39	.303	.0980
20	11.20	46.9	3.18	.239	.0678
21	9.00	33.1	2.56	.272	.0773

8 DAY EMBRYOS

Vial No.	(1) Micrograms of RNA	(2) Micrograms of DNA	(3) Milligrams of Protein	RNA/ DNA	Protein/ DNA
22	7.47	27.2	2.14	.275	.0787
23	8.30	51.8	3.53	.160	.0681
24	8.77	32.1	2.96	.273	.0922
25	8.40	49.4	3.35	.170	.0678
26	10.73	65.7	3.82	.163	.0581
27	10.64	33.1	2.75	.321	.0831
28	9.56	33.6	2.89	.285	.0860
29	9.10	29.7	2.68	.306	.0902
30	9.70	50.9	2.99	.191	.0587

12 DAY EMBRYOS

Vial No.	(1) Micrograms of RNA	(2) Micrograms of DNA	(3) Milligrams of Protein	RNA/ DNA	Protein/ DNA
1	9.30	45.0	2.69	.207	.0598
2	8.84	49.8	3.06	.178	.0614
3	9.21	41.3	2.83	.223	.0685
4	8.84	47.0	2.62	.188	.0557
5	7.71	43.1	2.51	.179	.0582
6	10.20	50.6	2.92	.202	.0577
7	8.84	44.6	2.38	.198	.0534
8	10.66	45.9	3.10	.232	.0675
9	9.75	43.7	3.03	.223	.0693
10	10.57	51.8	3.33	.204	.0643
11	8.62	44.0	2.58	.196	.0586
12	7.48	32.6	2.31	.229	.0709
13	7.48	31.7	1.94	.236	.0612
14	9.43	37.5	2.79	.251	.0744
15	8.39	36.0	2.76	.233	.0767
16	11.25	57.6	3.71	.195	.0644
17	9.66	40.2	2.96	.240	.0736
18	10.57	39.2	3.10	.269	.0789
19	11.02	45.6	3.26	.242	.0715
20	___*	45.6	2.92	___*	.0640
21	9.21	36.3	2.85	.254	.0785

*Sample Lost

12 DAY EMBRYOS (Con't)

Vial No.	(1) Micrograms of RNA	(2) Micrograms of DNA	(3) Milligrams of Protein	RNA/ DNA	Protein/ DNA
22	10.79	40.7	3.12	.265	.0767
23	7.94	35.6	2.79	.223	.0784
24	7.71	40.7	3.03	.189	.0744
25	8.53	37.8	2.92	.226	.0772
26	11.47	42.3	3.67	.271	.0868
27	9.89	46.2	3.23	.214	.0699
28	10.79	47.9	3.12	.225	.0651
29	10.20	46.2	3.03	.221	.0656

APPENDIX H

STATISTICAL PARAMETERS OF EACH ASSAY GROUP

MILLIGRAMS OF PROTEIN PER MICROGRAMS OF DNA

Group	Mean	Variance	Sample Size
A	.0454	.000111	4
B	.0525	.000110	4
C	.0588	.000109	5
D	.0466	.000001	5
E	.0503	.000008	5
F	.0475	.000020	5
G	.0566	.000245	5
H	.0874	.001085	5
I	.0657	.000216	5
J	.0941	.000291	5
K	.0768	.000081	5
L	.0752	.000198	5
M	.0607	.000018	5
N	.0624	.000036	5
O	.0684	.000052	5
P	.0705	.000032	5
Q	.0770	.000002	5
R	.0706	.000069	5

MICROGRAMS OF RNA PER MICROGRAMS OF DNA

Group	Mean	Variance	Sample Size
A	.182	.000962	4
B	.296	.004862	5
C	.265	.000195	5
D	.244	.000310	5
E	.245	.000085	5
F	.227	.000279	5
G	.183	.002789	5
H	.281	.010974	5
I	.209	.004889	5
J	.315	.005517	5
K	.230	.002828	5
L	.253	.004080	5
M	.195	.000303	5
N	.212	.000174	5
O	.229	.000328	5
P	.237	.000708	4
Q	.231	.000708	5
R	.228	.000493	5

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VITA

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Mr. Rivers became a student at the Georgia Institute of Technology in September 1961 and graduated in December 1965 with a Bachelor's degree in industrial engineering. He was a member of Alpha Epsilon Pi social fraternity.

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He is married to the former Stephanie Carr of Philadelphia, Pennsylvania and presently resides in Blacksburg, Virginia.